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CLIFTON, KELLY H. AND ROLAND K. MEYER
Effect of Food Intake on the Secretion of Thyrotrophin during Diethylstilbestrol Treatment 681

MEYER, ROLAND K. AND KELLY H. CLIFTON
Effect of Diethylstilbestrol-Induced Tumorigenesis on the Secretory Activity of the Rat Anterior Pituitary Gland 686

SHIRLEY, H. V., JR. AND A. V. NALBANDOV
Effects of Transecting Hypophyseal Stalks in Laying Hens 694

BAVETTA, L. A., S. BERNICK AND B. H. ERSHOFF
Selective Effects of Growth Hormone on Rats Fed a Tryptophan-Low Diet. 701

ROSEMBERG, EUGENIA, GEORGE ROSENFELD, FRANK UNGAR AND RALPH I. DORFMAN
Conversion of Steroids to Aldosterone-like Material 708

ELIAS, HANS AND JOHN E. PAULY
The Structure of the Human Adrenal Cortex 714

SELYE, HANS AND PIERRE BOIS
Indispensability of the Adrenals for the Nephrosclerotic Action of Hypophyseal Preparations 739

KORITZ, SEYMOUR B. AND RALPH I. DORFMAN
Effect of Hormones on the *In Vitro* Incorporation of Glycine-1-C^{14} into Reticulocyte Proteins 748

GILBERT, CHRISTINE AND JOSEPH GILLMAN
The Role of the Adrenal and Thyroid in Promoting Bleeding in Hypophysectomized Baboons (Papio ursinus) 753

GREENSPAN, FRANCIS S., JOSEPH P. KRISS, LINCOLN E. MOSES, AND WILLIAM LEW
An Improved Bioassay Method for Thyrotropic Hormone using Thyroid Uptake of Radiophosphorus 767

COLLINS, ELLIOTT J.
Steroid-Induced Adrenal-Pituitary Hypofunction 777

LYSTER, STANLEY C., GEORGE H. LUND AND ROBERT O. STAFFORD
Androgenic and Myotrophic Properties of Orally Administered 9-Fluoro-11-Oxy-Methyltestosterones 781

EVERETT, JOHN W.
Functional Corpora Lutea Maintained for Months by Autografts of Rat Hypophyses 786

BEATON, GEORGE H. AND DORIS M. CURRY
A Comparison of the Effects of Growth Hormone and of Insulin Administration 797

TULLNER, WILLIAM W., MORRIS M. GRAFF AND ROY HERTZ
Amphenone Inhibition of Adrenal Corticosteroid Output in the Hypophysectomized Dog 802

ENGEL, MILDRED G. AND FRANK L. ENGEL
Influence of Oxycel Corticotropin on Fatty Acid and Glucose Metabolism in the Rat 808

THE ENDOCRINE SOCIETY MEDAL, 1956 818

THE CIBA AWARD, 1956 819

PUBLIC HEALTH SERVICE 820

PLANNED PARENTHOOD FEDERATION OF AMERICA, INC. 820

INDEX 821

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ERRATA

In the paper entitled "The Antiluteinizing Potency of Five Derivatives of Progesterone" by Elvira Mardones, R. Iglesias and A. Lipschutz in the February, 1956 issue of ENDOCRINOLOGY.

Page 215, line 5 should read 11-β-OH-P, instead of 11-keto-P.

Page 215, line 6 should read 11-keto-P, instead of 11β-OH-P.

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EFFECT OF FOOD INTAKE ON THE SECRETION OF THYROTROPHIN DURING DIETHYLSTILBESTROL TREATMENT[1]

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ESTROGEN treatment has been variously reported to increase thyroid activity (Emge and Laqueur, 1941; Reineke and Soliman, 1953; Engstrom and Markardt, 1954), to have little effect on thyroid function (Paschkis, Cantarow and Peacock, 1948; Desclin and Ermans, 1951), and to inhibit thyroid cell growth and colloid resorption (Gardner, 1949; Tuchman-Duplessis and Mercier-Parot, 1953). Underfeeding reduces the rat thyroid response to thiouracil in proportion to the reduction in body growth (Meites and Agrawala, 1949). Meites (1949) showed that large doses of estrogen will reduce the appetite of rats and speculated as to whether the decrease in food intake was the cause of, or a result of, an inhibition of thyrotrophin secretion by the estrogen.

Diethylstilbestrol stimulates adrenocorticoid secretion (Fry, Miller and Long, 1942; Nadel, Josephson and Mulay, 1950) and cortisone has been reported to inhibit (Perry, 1951; Woodbury, Ghosh and Sayers, 1951) or stimulate (Spirtos, 1953) thyroid function. In the present report data are presented which show that the inhibition of thyrotrophin secretion occurring in rats treated with large doses of diethylstilbestrol is a result of the concurrent inanition. When inanition is precluded by force-feeding, diethylstilbestrol stimulates thyroid activity.

MATERIALS AND METHODS

Male rats obtained from the Holtzman Rat Company were used in all the experiments. The secretion of thyrotrophin was measured by the degree of compensatory thyroid hypertrophy over a 15 day period following partial thyroidectomy. The right thyroid lobe and half the isthmus was removed and weighed. Fifteen days later the animals were killed and the left half of the thyroid was removed and weighed. The increase in

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[2] Public Health Service Research Fellow of the National Cancer Institute (1951–1954). Present address: Children's Cancer Research Foundation, 35 Binney St., Boston.

the weight of the left thyroid half over that of the right half served as an index of the quantity of thyrotrophin secreted by the anterior pituitary gland.

In Experiments 1 and 2 both the diethylstilbestrol (DES) treated and control animals were maintained throughout on Rockland rat pellets fed *ad libitum*. In Experiment 1, partial thyroidectomy was performed on a group of animals which had been implanted subcutaneously with 15 mg. DES pellets[3] 70 days previously when the animals were 30 days of age, and in a group of untreated control rats of the same age. In Experiment 2 the partial thyroidectomy was performed at 30 days of age at the time of DES pellet implantation.

A diet modified from that described for force-feeding by Ingle (1953) was used in Experiments 3 and 4. The dry components of the diet were mixed in the proportions recommended by Ingle and stored in sealed containers. The oils were mixed in the suggested proportions and stored under refrigeration. For force-feeding, the diet was mixed in a Waring blendor in the proportion of 25 ml. mixed oils and 150 ml. water for every 100 gm. of the dry components.

In Experiments 3 and 4 force-feeding was started in all animals at 22 days of age, 8 days before the operation and initiation of DES treatment. The force-feeding volume was increased slowly from 1 ml. per animal. Rockland pellets were continued *ad libitum* until 2 days before the operation. In Experiment 3, 2 daily feedings were given until the twelfth day after the operation when a third feeding was added and continued until the termination of the experiment. The volume per feeding was slowly increased throughout the experiment.

On the day of partial thyroidectomy, the animals in Experiment 4 were divided into 4 groups; 2 groups received DES pellets and 2 groups served as controls. Force-feeding 3 times daily was continued in 1 each of the DES-treated and control groups. The other DES-treated and control groups were fed the oils and dry components of Ingle's diet mixed in the proportions used for force-feeding and given *ad libitum* for the duration of the experiment. The animals were weighed daily, and the force-feeding volume was adjusted to maintain the growth of the force-fed control animals as near as possible to that of the control animals fed *ad libitum*.

At the termination of Experiment 4, the pituitary glands of all of the animals were fixed in 9 parts SUSA to 1 part saturated picric acid. The fixed glands were weighed after the post-fixation water wash. The left thyroid halves were weighed and then fixed with their respective pituitary glands. The paraffin blocks were coded; the tissues sectioned at 4 μ and stained according to the method of Halmi (1952c). Three sections of each pituitary gland which were approximately equidistant from the outside surfaces and from each other were used in estimating the percentage of beta basophils. The total number of cells and of beta basophils was determined in every third oil-immersion field in a straight line through the longest axis of the section. Observations were made on the appearance of the beta cells and on the condition of the thyroid glands. The tissues were not decoded until completion of these determinations.

In Experiment 5, 13 30-day-old rats were partially thyroidectomized. Cortisone acetate[4] in fine suspension in corn oil was given to 6 of the animals at a dose of 2.0 mg. in 0.20 ml. for the first 5 days and 2.5 mg. in 0.25 ml. for the remaining 10 days of the experiment. Seven animals served as controls. Rockland rat pellets were given *ad libitum* to both the experimental and control groups, and the daily group food intake was recorded.

[3] Stil-Capo diethylstilbestrol pellets, American Scientific Laboratories, Madison, Wisconsin.

[4] Crystalline cortisone acetate, lot #50214, Merck and Company, Rahway, New Jersey.

RESULTS AND DISCUSSION

The feeding regimes, hormone treatments and results are summarized in Table 1 and Figure 1. It can be assumed that any effect of estrogen on thyroid hypertrophy is mediated through the anterior pituitary. Compensatory thyroid hypertrophy can thus be used as an index of the thyrotrophin secretion.

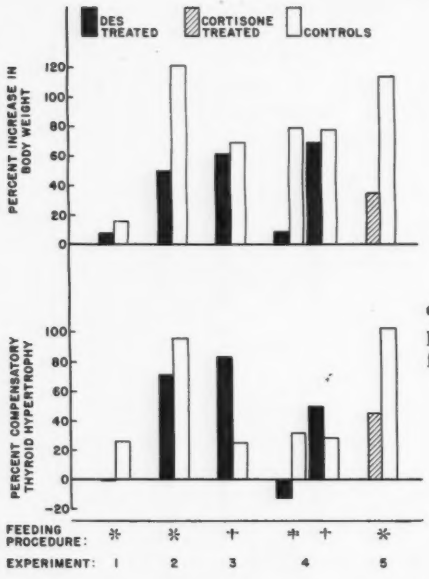
DES markedly reduced both body growth and compensatory thyroid hypertrophy in the animals in Experiments 1 and 2 which were fed Rockland pellets *ad libitum*, and in those in Experiment 4 fed the Ingle diet *ad libitum*. These two effects appear to be closely related (Fig. 1). DES stimulated thyrotrophin production in Experiments 3 and 4 when the body growth of the treated rats was maintained at approximately the same rate as that of the control animals by force-feeding.

The differences in thyrotrophin secretion between the groups in Experiment 4 were not accompanied by demonstrable differences in the percentages of pituitary beta basophils, the presumptive cell source of this hormone (Purves and Griesbach, 1951a, b and c; Halmi, 1952a and b) (Table 2). The beta cells and thyroids of the DES-treated group which was fed *ad libitum* did differ histologically, however, from those of the other three groups in the experiment. In the former group, the beta cells were predominantly small. The cytoplasm of such cells was either finely granulated or appeared as a basophilic ring around the nucleus. The thyroids displayed more colloid than those of the other three groups, though the chief cells were columnar and active colloid resorption was occurring in some of the follicles. Extensive colloid resorption was common in the thy-

TABLE 1. COMPARISON OF THE EFFECTS OF DIETHYLSTILBESTROL AND CORTISONE WITH VARIOUS FEEDING REGIMES

Experiment number	Treatment and number of animals	Feeding regime	Thyroid data		Body weight, gm.		Combined adrenal weight, mg.	Pituitary weight, mg.
			Right lobe weight, mg.	Left lobe minus right lobe weight	Initial	Final		
1	8 DES	Rockland *ad lib.*	4.81±0.67[1]	−0.06±0.27	137±11	149±13	—	13.3±3.1
	8 Controls		6.84±0.70**	1.77±0.96**	305±17**	352±26**	—	10.3±0.8*
2	7 DES	Rockland *ad lib.*	2.87±0.23	2.03±0.33	69± 4	105± 7	—	7.4±1.4
	7 Controls		2.84±0.20	2.69±0.54*	68± 3	150± 9**	—	5.9±0.7*
3	8 DES	Ingle Force-Fed	2.86±0.53	2.36±0.76	74± 3	120± 4	—	7.5±0.9
	7 Controls		3.12±0.38	0.78±0.20**	74± 3	124± 6	—	5.1±0.3**
4	7 DES	Ingle *ad lib.*	3.21±0.56	−0.41±0.46	78± 5	84±10	22.8±2.4	5.5±1.0
	7 Controls		3.21±0.60	0.99±0.32**	77± 5	139±11**	26.1±3.0*	5.5±0.5*
	4 DES	Ingle Force-Fed	3.36±0.36	1.64±0.21	75± 4	128± 4[2]	39.2±7.8	9.0±1.4
	5 Controls		3.21±0.32	0.91±0.31**	75± 4	134± 3	32.4±2.4	4.8±0.6**
5	6 Cortisone	Rockland *ad lib.*	2.62±0.44	1.19±1.02	72± 4	97±17	11.3±1.2	—
	7 Controls		2.58±0.20	2.63±1.06*	73± 5	156±22**	29.5±1.7**	—

[1] All values presented ±standard deviation.
[2] Different from force-fed controls at P .05 level, but insignificantly different from controls fed *ad libitum*.
* Difference between treated and control groups significant at P .05 level.
** Difference between treated and control groups significant at P .01 level.



* Rockland rat pellets fed *ad libitum*.
† Ingle's diet, force-fed.
‡ Ingle's diet fed *ad libitum*.

FIG. 1. Effect of diethylstilbestrol and cortisone on body weight increase and compensatory thyroid hypertrophy with different feeding regimes.

roids of the other three groups, and the chief cells were columnar. The beta cells of these groups were similar, either of normal size and well granulated or enlarged and partially degranulated.

The effect of DES on the pituitary-thyroid system does not appear to be mediated through the adrenal cortices. Exogenous cortisone acetate reduced both body growth and compensatory thyroid hypertrophy in Experiment 5. This effect was not the result of inanition. The cumulative average food intake in the experiment was 186.9 gm. per control animal and 187.0 gm. per cortisone-treated animal. Adrenal weight showed no clear-cut relation to the thyroid response in Experiment 4. The DES-treated, force-fed animals bore the heaviest adrenals and displayed the greatest compensatory thyroid hypertrophy. The adrenals of the force-fed control rats were significantly heavier than those of their counterparts which were fed *ad libitum*, but the thyroid responses of the two groups were nearly identical.

TABLE 2. THE PERCENTAGE OF BETA CELLS IN THE PITUITARY GLANDS OF THE ANIMALS IN EXPERIMENT 4. TWO THOUSAND TO 3500 NUCLEI WERE COUNTED PER GLAND

Treatment	Number of glands counted	% beta basophils
Controls, force-fed	5	2.03 (1.33–2.73)[1]
DES-treated, force-fed	4	1.44 (0.71–2.21)
Controls, fed *ad libitum*	7	1.57 (0.40–2.85)
DES-treated, fed *ad libitum*	6	1.74 (0.70–2.53)

[1] Range in parentheses. t-test for significance of difference between groups was performed after Bliss' angle transformation which corrects for skewing of proportions (Snedecor, 1946). No significant differences found between groups.

DES thus appears to stimulate thyrotrophin secretion directly. Estrogen has been reported to counteract the calorigenic action of thyroid hormone (Sherwood, 1941). It is suggested that high estrogen levels antagonize the effect of thyroid hormone on a thyrotrophin controlling center as well as on the peripheral tissues. High estrogen would then cause an increase in thyrotrophin and thyroid hormone production without marked calorigenic effects. Such a mechanism would maintain a high gradient of thyroid hormone at the placenta during pregnancy, and thereby increase its availability to the fetus.

SUMMARY

Diethylstilbestrol stimulates compensatory thyroid hypertrophy in rats when an adequate food intake is maintained by force-feeding. The inhibition of body growth and compensatory thyroid hypertrophy which occurs in diethylstilbestrol-treated rats that are fed *ad libitum* is a result of reduced appetite and consequent inanition.

The stimulation of compensatory thyroid hypertrophy by diethylstilbestrol does not appear to be mediated through the adrenals. It is suggested that estrogen causes an increase in thyrotrophin secretion by the anterior pituitary directly, and a mechanism is postulated for this effect.

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EFFECT OF DIETHYLSTILBESTROL-INDUCED TUMORIGENESIS ON THE SECRETORY ACTIVITY OF THE RAT ANTERIOR PITUITARY GLAND[1]

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PROLONGED estrogen treatment results in tumorigenesis of the anterior pituitary glands of rats and mice (Zondek, 1936; McEuen, Selye and Collip, 1936; Cramer and Horning, 1936). Such tumors do not arise until several months after the initiation of treatment and terminate in the death of the animal as a result of increased intracranial pressure (Nelson, 1941). Though the tumors have been studied extensively in the last two decades, the mechanism of induction has remained obscure.

Several authors have proposed an association between the tumorigenic action and the effects of estrogen on pituitary secretory activity (Zondek, 1941; Gardner, 1953; and Furth, 1953).

In experiments of short duration, estrogen has been reported to inhibit the secretion of gonadotrophins (Byrnes and Meyer, 1951), to stimulate the secretion of prolactin (Meites and Turner, 1948) and adrenocorticotrophin (Fry, Miller and Long, 1942; Nadel, Josephson and Mulay, 1950), and to inhibit body growth (Zondek, 1941). If an adequate food intake is maintained, estrogen stimulates thyrotrophin secretion (Clifton and Meyer, 1956a).

Zeckwer (1944), Lacour (1950) and Bielschowsky (1954) noted milk-filled cysts in the mammary ducts of rats bearing estrogen-induced tumors, and concluded that tumorigenesis is the result of stimulation of the prolactin-secreting cells. The present investigations were designed to test this hypothesis and to further clarify the relationship between pituitary tumorigenesis and the effects of prolonged estrogen treatment on the secretion of the other pituitary hormones.

MATERIALS AND METHODS

Several groups of 30-day-old male rats obtained from the Holtzman Rat Company were implanted subcutaneously with pellets[3] containing 15 mg. diethylstilbestrol (DES).

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[2] Public Health Service Research Fellow of the National Cancer Institute (1951–1954). Present address: Children's Cancer Research Foundation, 35 Binney Street, Boston, Mass.

[3] Stil-Capo diethylstilbestrol pellets, American Scientific Laboratories.

New pellets were implanted every 3 or 4 months. All groups of treated animals were accompanied by untreated controls of the same age, and fed Rockland rat pellets *ad libitum*. Pituitary tumors were found in this strain only after 9 or more months of treatment (Clifton and Meyer, 1956b).

Autopsies were performed on a total of 87 DES-treated and 67 control animals killed in groups at 10, 20, 30, 40, 60, 90, 147 and 210–466 days after the initiation of treatment. Anterior pituitary tissue to be used in assays was removed immediately after death, weighed and homogenized in chilled distilled water or saline, or if to be assayed later, was frozen on dry ice and stored in a freezing compartment. All pituitary tumors used in assays weighed in excess of 100 mg.

Prolactin: Assays of tumorous and normal pituitary tissues were performed by a modification of the method of Reece and Turner (1937) in 6 to 8-week-old White King squab. Four daily intradermal injections were given over each side of the crop. Each bird received an homogenate of tumor tissue over one side and normal pituitary tissue at the same dose over the other side. The sides receiving tumor and normal tissue were alternated at each dose level. In the first assay series, total doses of 1.25, 2.50, 5.0, 10.0, 20.0 and 40.0 mg. pituitary tissue were given per side per bird. In the second series, total doses of 10.0 and 30.0 mg. were given per side per bird.

The birds were coded and killed 24 hours after the last injection. Measurements of crop gland diameter were made without knowledge of the dosage or tissue the bird had received.

Gonadotrophins: The first of a series of 3 gonadotrophin assays was performed in Holtzman male rats hypophysectomized at 30 days of age. The animals were injected subcutaneously twice daily with homogenates of tumorous or normal anterior pituitary tissue or with saline placebo for a period of 10 days beginning on the fourth day after operation. The testes and sex accessories were weighed at autopsy the morning after the last injection.

The luteinizing hormone (LH) potency of pituitary tissue from control and DES-treated rats was further tested in the second and third gonadotrophin assays. The treated pituitary tissue in the second assay was from animals which had received DES for 9 months, but in which tumorous pituitary growth had not occurred. In the third assay, DES-induced pituitary tumor tissue was tested. Holtzman female rats, 22 days of age at the time of the first injection were used in both assays. Six equal subcutaneous injections of placebo, purified follicle-stimulating hormone[4] (FSH), or FSH and homogenized tissue were given over 3 days. The ovaries were weighed and inspected for luteinization at autopsy on the fourth day.

Growth-promoting factors: Tumorous and normal pituitary tissue were assayed by the tibial line method of Evans, Simpson, Marx and Kibrick (1943). Holtzman female rats, hypophysectomized at 24 or 25 days of age, were given 5 equal daily intraperitoneal injections of homogenized tissue or saline placebo beginning at 38 days of age. The tibia were removed, split and fixed at autopsy the day following the last injection, and the fixed bones coded. Measurements of the tibial line width were performed after staining without knowledge of the treatment the animals had received.

Two types of tumorous pituitary tissue were assayed in this series. Tumor type I was from intact animals treated with DES for in excess of 9 months. Tumor type II was from a group of animals which had been surgically thyroidectomized as well as treated with DES.

Pituitary-adrenal relationship: The adrenals of groups of control rats and rats treated

[4] FSH #S67A prepared from sheep pituitary powder by Dr. W. H. McShan. 7.22 mg. preparation from 1 gm. starting material.

with DES for 10, 20, 30 and 40 days and in excess of 9 months were fixed in formalin, imbedded in gelatin, sectioned at 15 μ and stained with sudan orange or sudan black B. The cortical lipid content and distribution in such sections was studied as an indication of the rate of adrenal secretory activity.

The ability of the anterior pituitary to secrete adrenocorticotrophin (ACTH) during the administration of DES was estimated in a series of three experiments. The left adrenals of groups of DES-treated and control animals were removed. The right adrenal glands were brought to the body surface, the capsules slit and the cortical and medullary tissues extruded with forceps. The empty capsules were returned to the body cavities. Fifteen days later the animals were killed and the right adrenal capsules and regenerated cortices removed and weighed. The animals were given a choice of tap water or 0.9% saline *ad libitum* during the experimental period.

Pituitary-thyroid relationship: The ability of DES-induced anterior pituitary tumors to induce compensatory thyroid hypertrophy was compared to that of untreated control animals. The right thyroid lobes and half the isthmi were removed and weighed. Fifteen days later, the animals were killed and the left thyroid lobes and remaining isthmi removed and weighed. The increase in weight of the left thyroid lobe and isthmus over that of the right thyroid lobe and isthmus was used as an estimate of the secretion of thyrotrophin (TSH) during the experimental period.

RESULTS

Prolactin: Duct cysts measuring several millimeters in diameter and containing a milky fluid were a constant finding in the mammary areas of tumor-bearing rats. Smaller milky cysts were present in nine of ten animals autopsied at sixty days of DES administration, six months before the onset of rapid pituitary tumor growth. Pituitary tumor tissue was found to be slightly more potent in prolactin activity per mg. wet weight than normal pituitary tissue in the first assay series (Fig. 1). The results of the second assay were similar. An entire tumor weighing between 30 and 400 mg. would thus contain from three to forty times the prolactin potency of a whole normal gland. The results are in agreement with the conclusions of Zeckwer (1944), Lacour (1950) and Bielschowsky (1954). Prolactin secretion is stimulated during the first few months of DES administration, and the concentration of the hormone in pituitary tissue of treated rats is maintained at nearly normal levels despite rapid tumorous growth.

Gonadotrophins: Significant atrophy of the testes and sex accessories was evident in experimental animals by ten days of DES administration. No gonadotrophin was demonstrated by assay in the pituitary tissue of DES-treated animals. Normal anterior pituitary tissue induced growth of the testes and sex accessories of hypophysectomized male rats (Table 1), and caused luteinization and hypertrophy of the ovaries of immature females when given with purified FSH (Table 2). No indications of luteinization were found in the ovaries of females injected with tumorous or nontumorous, DES-treated pituitary tissue in conjunction with FSH.

Growth-promoting factors: DES markedly inhibited body growth throughout the tumor induction period. In the tibial line assay, normal

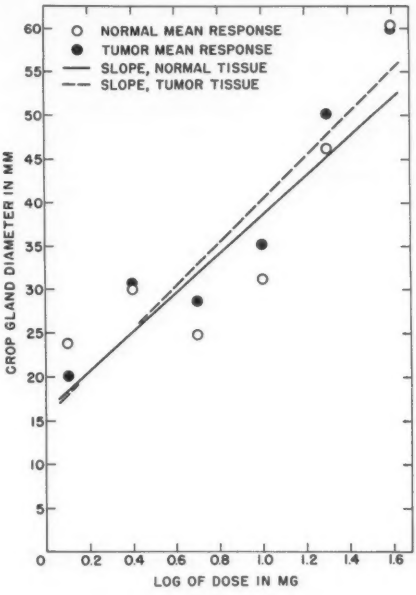

FIG. 1. Prolactin assay of normal and tumorous pituitary tissue. Tumor tissue potency is 115% of normal with a range of 42 to 319% according to statistical analysis by Irwin's method as outlined by Pugsley (1946).

pituitary tissue induced a significant response at both doses (Table 1). DES-induced tumor tissue of type I did not induce tibial line growth at either dose. Tumor tissue of type II induced a significant increase in tibial line width at both doses, but the greater dose resulted in the lesser response.

Several hormonal substances including TSH and prolactin will induce a "non-specific" increase of up to 20% in the width of the tibial plate. ACTH antagonizes growth hormone (GH) in the assay (Greenspan, Li, Simpson and Evans, 1950). The results in the present study therefore represent the

TABLE 1. GROWTH-PROMOTING AND GONADOTROPHIC POTENCIES OF TUMOROUS AND NORMAL PITUITARY TISSUES IN HYPOPHYSECTOMIZED RATS

Pituitary		Tibial line width in arbitrary units	Testes weight, mg.	Ventral prostate weight, mg.
Tissue type	Dose in mg.			
None	—	12.7 ± 1.9[1] (5)[2]	215 ± 40 (4)	8.4 ± 1.4
Normal	10.0	18.1 ± 0.8† (5)	896 ± 124† (4)	35.4 ± 10.4†
Normal	40.0	20.4 ± 1.1† (5)	1012 ± 37† (4)	94.1 ± 10.4†
Tumor Type I	10.0	15.0 ± 2.3 (5)	322 ± 76 (2)	9.8 ± 0.6
Tumor Type I	40.0	13.2 ± 0.5 (5)	234 ± 55 (3)	8.6 ± 2.3
Tumor Type II	10.0	18.5 ± 1.7† (4)	—	—
Tumor Type II	40.0	16.6 ± 1.9* (5)	—	—

[1] Values given ± standard deviation.
[2] Number of assay animals.
* Significantly greater than value of group not receiving tissue at P.05.
† Significantly greater than value of group not receiving tissue at P.01.

TABLE 2. COMPARISON OF THE FSH AUGMENTATION POTENCIES OF NORMAL AND DIETHYLSTILBESTROL-TREATED TUMOROUS AND NON-TUMOROUS PITUITARY TISSUES

Number of assay animals	FSH dose in mg.	Pituitary		Ovary weight in mg.
		Tissue type	Dose in mg.	
Series II				
5	0	None	—	14.8 ± 2.2[1]
10	3.61	None	—	40.1 ± 6.4
10	3.61	Normal	4.5	102.7 ± 19.8[2]
10	3.61	DES-treated, non-tumorous	4.5	41.7 ± 7.7[3]
Series III				
5	0	None	—	15.2 ± 1.5
8	4.33	None	—	31.9 ± 1.3
4	4.33	Tumorous	10.0	31.3 ± 3.4[3]
4	4.33	Tumorous	40.0	34.8 ± 3.8[3]

[1] Values given ± standard deviation.
[2] Augmentation significant at P.01.
[3] No significant augmentation of FSH action.

effects of any GH present plus the antagonistic and synergistic actions of any of these other hormones present. Although the data are therefore not conclusive, they are suggestive that the GH concentration in DES-induced tumor tissue is less than that in normal pituitary tissue. It cannot be determined from the present data whether DES actually inhibits GH synthesis and release.

Pituitary-adrenal relationship: The secretion of ACTH was found to be accelerated by DES administration in agreement with previous authors. Tumorigenesis neither accelerated nor inhibited its release in the present experiments. Despite retarded body growth the adrenal glands of the DES-treated rats were at least as heavy as those of their respective control animals throughout the tumor induction period. The cortices of DES-treated animals showed a progressive loss of sudanophilic material from ten through forty days of treatment. Fifteen of the nineteen adrenals examined from pituitary tumor-bearing rats showed normal sudanophilia, and the

TABLE 3. ADRENAL CORTEX REGENERATION AT VARIOUS INTERVALS DURING CHRONIC DIETHYLSTILBESTROL ADMINISTRATION

Number of animals and group	Days of treatment	Regenerated right cortex and capsule weight, mg.	Pituitary weight in mg.	Final body weight in gm.
7 DES-treated	85	18.94 ± 8.43[1]	12.6 ± 2.6*	172 ± 20†
6 Controls		13.93 ± 2.77	10.1 ± 1.0	349 ± 25
5 DES-treated	196	23.09 ± 3.20	19.1 ± 4.9†	160 ± 9†
7 Controls		19.57 ± 7.49	11.2 ± 1.4	393 ± 40
4 DES-treated	350	18.44 ± 3.62	128.7 ± 69.4†	197 ± 13†
6 Controls		19.49 ± 7.88	12.0 ± 1.2	470 ± 32

[1] All values given ± standard deviation.
* Difference between treated and control values significant at P.05.
† Difference between treated and control values significant at P.01.

TABLE 4. COMPENSATORY THYROID HYPERTROPHY IN RATS BEARING DIETHYLSTILBESTROL-INDUCED TUMORS OF THE ANTERIOR PITUITARY GLAND

Number of animals and group	Right thyroid weight, mg.	Left thyroid minus right thyroid weight in mg.	Pituitary weight in mg.	Final body weight in gm.
7 DES-treated	5.96 ± 0.53†	1.14 ± 1.14*	123.8 ± 77.5†	195 ± 25†
7 Controls	10.41 ± 1.68[1]	2.39 ± 0.97	12.4 ± 1.8	505 ± 27

* Difference between treated and control values significant at P .05.
† Difference between treated and control values significant at P .01.
[1] All values given ± standard deviation.

lipid material was but partially depleted in the adrenals of the remaining four. Adrenal regeneration was found to proceed at least at a normal rate in DES treated animals, even after the initiation of rapid tumorous proliferation (Table 3).

Pituitary-thyroid relationship: DES-induced pituitary tumorigenesis did not result in a pronounced increase in TSH synthesis and release. In terms of absolute weight, the compensatory thyroid hypertrophy was only half as great in the tumor-bearing animals as in the controls (Table 4). The percentage increase in the weights of the left thyroid lobes and isthmi over that of the right lobes and isthmi were, however, nearly the same in the two groups. The remaining thyroid tissue of the treated animals hypertrophied 19% while that of the control animals hypertrophied 23% during the fifteen day experimental period.

DISCUSSION

Estrogen-induced pituitary tumorigenesis is associated with the hypersecretion of prolactin as first postulated by Zeckwer (1944). The cells secreting other pituitary hormones do not seem to be directly involved in the tumorigenic process. There is no evidence in the present experiments which indicates a relationship between the inhibition of FSH and LH secretion and the pituitary tumor induction. The adrenal weight, lipid distribution and regeneration data in the DES treated animals are consistent with the description of an alarm reaction followed by a period of adaptation (Sayers and Sayers, 1948), and the response is not affected by tumorigenesis. Clifton and Meyer (1956a) found that DES inhibited the secretion of TSH during the first fifteen days of treatment in rats fed *ad libitum*, a result of concurrent inanition. When an adequate food-intake was maintained by force-feeding, DES stimulated TSH production. The TSH-inhibitory effect of inanition is apparently counterbalanced by the direct stimulatory action of DES at the time of tumor formation. Compensatory thyroid hypertrophy occurs at nearly the same rate in tumor-bearing and control animals.

The effect of force-feeding on the secretion of pituitary hormones other than TSH was not investigated. It seems probable, however, that the pat-

tern of events leading to DES-induced tumorigenesis would be basically unaltered; i.e., the specific stimulation of prolactin-secreting cells by DES would still occur. The inhibition of LH and FSH secretion by DES does not appear to result from inanition; gonadotrophin inhibition has been demonstrated with physiologic doses of estrogen (Byrnes and Meyer, 1951).

Prolactin is generally thought to be a product of the acidophils (Pearse, 1952). In cytological studies performed in conjunction with the present experiments, enlarged acidophils comprised from 12 to 54 per cent of the cells in the DES-induced tumors. The remaining tumor cells were predominantly enlarged and non-granular. Foci composed of both these cell forms were common in the pituitaries of treated animals by the time the first milk cysts were noted at autopsy. Consideration of these and other data led to the conclusion that tumorigenesis is the result of the prolonged maintenance of a normal mitotic response to estrogen in the prolactin-secreting cells (Clifton and Meyer, 1956b). According to this hypothesis the rate of division during exposure to estrogen is in slight excess of that required to replace dying cells. Such mitotic activity maintained by constant estrogenic stimulation eventually leads to tumorous growth. No abnormal action of estrogen need be postulated to account for tumor induction. The prolactin secreting cells are innately capable of responding to stimulation by estrogen with division as well as secretion.

Pituitary tumors have been developed by other experimental procedures known to induce the hypersecretion of hormonal products. Radiothyroidectomy in mice results after many months in tumors which hypersecrete TSH (Furth and Burnett, 1951). These tumors develop from the beta basophils or "thyrotrophs" (Halmi and Gude, 1954). Tumors composed of basophils have been reported in mice many months after gonadectomy (Dickie and Woolley, 1947), a procedure which results in the hypersecretion of gonadotrophins. These observations imply that in the pituitary gland, as in the thyroid and adrenal, stimulation to division is concomitant with stimulation to secretion. The accelerated mitotic rate which results from intense stimulation perhaps increases the probability of malignant and autonomous change such as has been noted in the radiothyroidectomy induced tumors of mice.

SUMMARY

The secretion of prolactin is stimulated by chronic estrogen administration. This hormone is maintained at approximately normal concentration in the rapidly proliferating tumor tissue. The secretion of adrenocorticotrophin occurs at an accelerated rate during the chronic administration of diethylstilbestrol, and is not modified markedly by tumorous pituitary growth. Compensatory thyroid hypertrophy occurs at almost the same rate in tumor-bearing and control animals. The secretion and storage of both

follicle-stimulating and luteinizing hormones is inhibited during diethylstilbestrol administration. Tibial line assays suggest that the growth hormone concentration in tumor tissue is less than in normal pituitary tissue.

It is suggested that pituitary tumorigenesis is the result of the continual and prolonged maintenance of a normal mitotic response to estrogen in the prolactin-producing cells. The cells which produce the other pituitary hormones do not seem to be involved in the tumorigenic process.

Acknowledgment

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EFFECTS OF TRANSECTING HYPOPHYSEAL STALKS IN LAYING HENS[1]

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THE interrelationship between the anterior lobe of the hypophysis and the hypothalamus has been demonstrated experimentally in mammals by Harris (1950) and others. Harris (1950), among others, has shown that when the cut edges of the hypophyseal stalk are prevented from regenerating, normal reproductive activity is impaired or completely halted. Because of the mounting evidence of neurohumoral control of the adenohypophysis in chickens (Huston and Nalbandov, 1953; van Tienhoven, Nalbandov and Norton, 1954) it became of interest to determine the effects of interrupting the neural and humoral connections between the hypothalamus and the pituitary gland on the reproductive cycle of laying hens. After this study was in progress Benoit and Assenmacher (1953) published their work on the effects of this operation on the testes of ducks. They found that in birds the portal vessels do not form part of the infundibular stalk as they do in mammals. When they resected the infundibular stalk the testes of illuminated ducks enlarged in size but they failed to do so when the median eminence or the portal tract (carrying the portal vessels) was cut. They conclude that in ducks the hypophysis no longer secretes gonadotrophic hormone if it is deprived of blood coming from the median eminence.

METHODS

The birds from which the posterior lobe was to be removed were anesthetized with intravenous nembutal injection and were secured on the operating board in such a way as to prevent head movement. An incision was made alongside the trachea and lateral to the glottis exposing the buccal cavity. At a point just anterior to the transverse vein, a small incision was made through the mucosa and it and the underlying mucles were retracted exposing the basi-temporal bone. A piece of this bone measuring about one-half centimeter in diameter was excised. With the aid of a dental drill and a bone pick the cancellous bone was removed exposing the caudal end of the sella turcica. With the aid of a microscope, the portion of the bony shell surrounding both the posterior lobe and the caudal end of the anterior lobe of the pituitary was carefully removed. A needle was used to slit the dura mater along the slight depression indicating the junction of the two

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lobes. By lifting the caudal end of the anterior lobe upward the infundibular stalk was exposed. A piece of plastic with a cutting edge was then inserted between the two lobes and pushed anteriorly thus severing all connections between the anterior lobe, the median eminence and the posterior lobe. The bits of plastic used for this purpose measured approximately 5×1.5 mm and were 0.1 mm thick. If this bit of plastic was placed correctly and if it remained in place, it caused a complete and continuous separation of the anterior lobe from the rest of the pituitary gland. To ascertain the exact position of the plate, all birds were killed at the end of the experiment (70 days after the operation), and the sella turcica was removed and sectioned serially after proper fixation and decalcification. At the time of autopsy the ovary, oviduct, thyroids and adrenals were weighed and preserved in Bouin's solution for sectioning.

Because of the intricacy of the operation and the smallness of the operative site, it was not always possible to be certain of the exact location of the plastic piece. Neither was it possible to be certain that the stalk had been severed, that no regeneration had occurred and that the adenohypophysis was completely isolated from the hypothalamus. For these reasons it was thought best to check on the successfulness of the operation post-mortem, and to classify the birds subsequent to microscopic examination of the hypophyseal area. After serial sections of that area were made (at 10 μ) and examined, the 21 hens used in this experiment were divided into the groups according to the success of the operation.

EXPERIMENTAL RESULTS

Group A. Nine hens in which the hypophyseal portal system was completely transected and the entire anterior lobe of the pituitary was isolated by the plastic piece (complete stalk section, Fig. 1).

Group B. Six hens in which the plastic piece had cut through most of the stalk but in which a small portion of the anterior lobe of the pituitary was still connected with the portal vessels (almost complete stalk section, Fig. 2).

Group C. Six hens in which the plastic block had penetrated the anterior lobe leaving a sizeable portion of the gland connected to the portal system (incomplete stalk section, Fig. 3).

The operation resulted in a drastic and immediate reduction in body

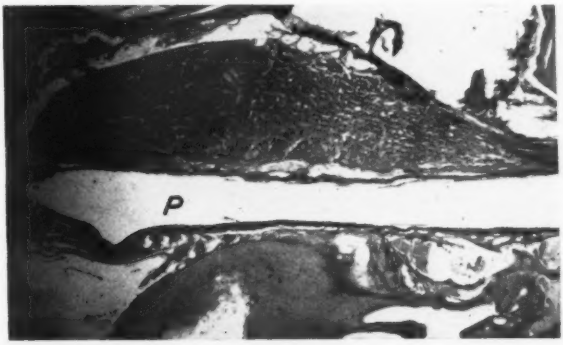

FIG. 1. Sagittal sections through the sella turcica showing the degree of separation of the anterior lobe from the hypothalamus. Group A. Complete stalk section.

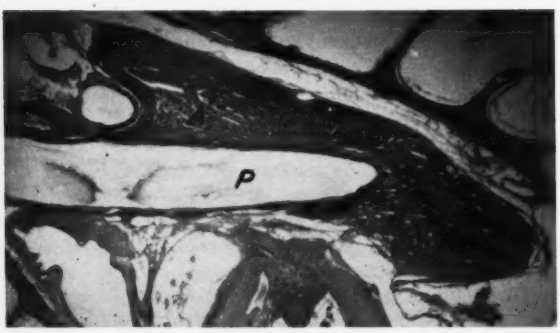

FIG. 2. Group B. The plastic piece (P) has cut through most of the stalk but a piece of hypophyseal tissue (A_1) remains connected with portal vessels.

weight of all three groups. However, birds with incompletely sectioned stalks (Group C), eventually regained and exceeded their preoperative weights. The birds in the other two groups eventually plateaued at about 75% of their preoperative weights (Fig. 4).

In all three groups ovulation and oviposition was completely stopped immediately following the operation. None of the females in which the stalks had been completely or almost completely sectioned (Groups A and B), ovulated or laid eggs during the 70-day observation period. In contrast, Group C in which the stalk section was incomplete, resumed laying about 36 days after the operation and laid at a rate comparable to that of the preoperative period (Table 1).

Further indication that complete or near complete stalk section leads to an impairment of function of the adenohypophysis was obtained from the fact that comb size decreased drastically following the operation and eventually stabilized itself at 26 and 32% of normal in Groups A and B but returned to normal in Group C (Fig. 5). Combs of females in Groups

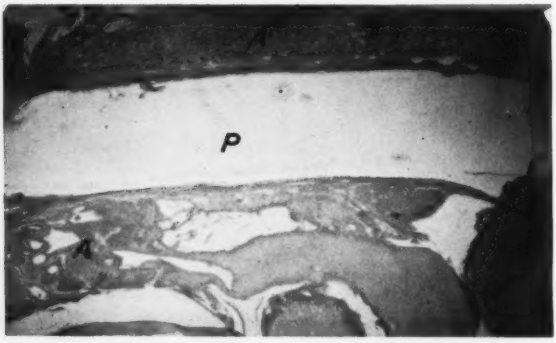

FIG. 3. Group C. Plastic piece (P) has penetrated the anterior lobe and cut it into two portions (A and A); incomplete stalk section.

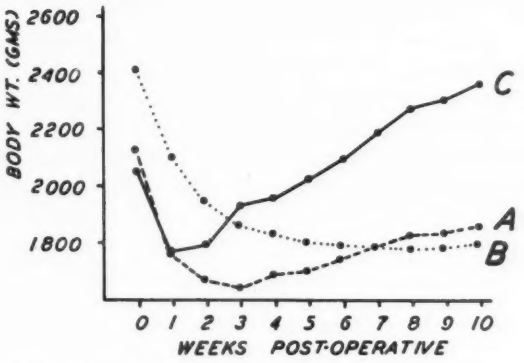

FIG. 4. Effect of stalk transection on body weight of hens A and B, complete and almost complete; C incomplete transection.

A and B resembled in size, color and texture those normally found in hypophysectomized hens.

Autopsy further showed that the ovaries of hens in Groups A and B had completely regressed being very significantly smaller than those of the females in Group C (Table 1). In both weight and degree of follicular development the ovaries of the females in Groups A and B resembled the ovaries of hypophysectomized females. That the ovaries of Groups A and B were not secreting androgen was shown by the fact that the combs were dry and greatly diminished in size. That the ovaries were not secreting estrogen was shown by the greatly diminished oviducal weights (Table 1).

The weights of the thyroid glands were somewhat smaller in Groups A and B while the adrenals were heavier in these two groups when compared to Group C. However, none of these differences was significant. Careful histological examination of these glands showed an essentially normal histological picture with none of the degenerative changes usually asso-

TABLE 1. EFFECT OF COMPLETE AND INCOMPLETE STALK SECTION ON REPRODUCTION AND ORGAN WEIGHTS OF HENS

	No. of hens	Days from operation to first egg (±SD)	Average daily egg production (±SD)	Average size of follicles at autopsy (cm.±SD)	Weight of				
					Ovary gm. ±SD	Oviduct gm. ±SD	Body gm. ±SD	Thyroids mgs. ±SD	Adrenals mgs. ±SD
Group A			did not lay						
Complete stalk section	9			0.4±0.2	1.37 ±0.23	3.52 ±1.27	1870 ±544	183±109	183±53
Group B			did not lay						
Almost complete stalk section	6			0.4±0.4	2.17 ±1.17	4.77 ±3.02	1801 ±476	151± 41	182±51
Group C									
Incomplete stalk section	6	35.8 ±14.1	0.43 ±0.22	2.7±0.5	36.08 ±7.20	46.42 ±7.27	2375 ±251	202± 81	149±26

ciated with hypophysectomy being apparent (Nalbandov and Card, 1943).

Histological examination of the anterior lobes of pituitary glands from Groups A and B showed profound changes. Most striking of these was the pronounced increase in fibrotic tissue as well as a decrease in the number of all the usual cell types. More careful analysis of the histology of these glands is necessary before a more detailed description of the changes is made. The histology of the hypophyses of birds in Group C was normal.

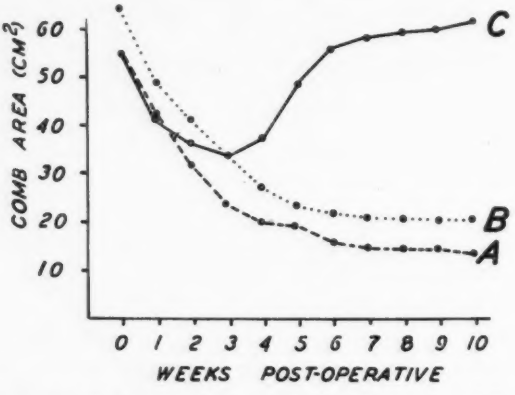

FIG. 5. Effect of stalk transection on comb size of hens. A and B, complete and almost complete; C incomplete transection.

DISCUSSION

In general the results of this work support the conclusions of those who have made similar studies on mammals. Complete interruption of the portal blood supply in chickens leads to impaired function of the anterior pituitary gland. In fact, in laying hens, stalk section produces a condition indistinguishable from hypophysectomy as far as the ovary and the sex hormone-dependent structures are concerned. Also reminiscent of hypophysectomy is the loss of weight which is not regained in birds in which stalk section is complete.

In several respects, however, stalk section produces results distinctly different from those observed following hypophysectomy. After hypophysectomy chickens require special environmental conditions such as constant high temperatures and night feeding. The latter prevents hypoglycemia and eventual death both of which usually result from the long nocturnal fast which hypophysectomized chickens can not tolerate. This is prevented in this laboratory by providing two lighted periods during the night when birds can eat. In contrast, birds with transected stalks were quite able to withstand sudden and drastic changes of temperature as well as the nocturnal fast without becoming moribund. While hens after hypophysectomy undergo an immediate and total body molt, hens with cut

stalks did not differ in their molting pattern from normal birds. Neither the weight nor the histology of the thyroids or the adrenals was altered by stalk section while hypophysectomy produced drastic changes in both the weight and the histology of these glands (Nalbandov and Card, 1943). Thus it appears that only the secretion of the gonadotrophic complex and possibly of the growth hormone is halted or at least drastically impaired by stalk section, while little or no change in the secretion of ACTH and the thyrotrophic hormone is observed. This agrees with the observations of Benoit and Assenmacher (1953) on ducks but is in contrast to the results obtained by Barrnett and Greep (1951) and Greep and Barrnett (1951) who observed a general hypopituitarism in a good share of rats in which stalk section was done. However, in both male and female rats the gonads, and the gonad-dependent end organs, showed some activity indicating that the secretion of hypophyseal gonadotrophins was decreased but not completely halted by stalk section. These workers explain pituitary hypofunction as being due to ischemia resulting from the interruption of the portal vascular system. Even though in our case profound histologic changes were observed in the pituitary gland, only the gonadotrophic complex and possibly growth hormone were affected by the operation, while ACTH and thyrotrophin continued to be secreted in sufficiently high amounts to prevent regression of thyroid and adrenal glands. It is also considered significant that stalk section does not affect the ability of subjects to withstand temperature changes and prolonged nocturnal fasts in spite of the fact that the trauma involved in stalk section is more severe than that involved in hypophysectomy. This further underscores the contention that, unlike hypophysectomy, stalk transection does not produce pan-hypopituitarism. These results are in accord with Harris' (1950) interpretation who ascribes gonad atrophy to interruption of flow of the hypothalamic neurohumor which may control gonadotrophic activity, and with the findings of Benoit and Assenmacher (1953) who find that the operation in question causes primarily an impairment of the secretion of gonadotrophic hormone. They also consider the blood supply to the anterior pituitary from other sources adequate to maintain the gland (in most cases) in an essentially normal condition.

Finally, attention is directed to the finding that in Group C, in which the stalk was incompletely sectioned, 35.8 days were required from the time of operation until ovulation and oviposition were resumed. In neurohypophysectomized hens in which the stalk was cut but in which no plastic plate was used to prevent regeneration of the stalk, 36.5 days elapsed between the operation and the first subsequent egg (Shirley and Nalbandov, 1956). In contrast, sham operated birds which were subjected only to the trauma of the operation, resumed ovulations 15.4 days after the operation. It is probably no mere coincidence that about 37 days passed between the operation and resumption of full ovarian activity in both

types of operations in which stalk transection was done. It is possible that about that length of time is required to regenerate the stalk and to reestablish the neurohumoral interrelation between the hypothalamus and the pituitary gland and to reestablish complete ovarian activity.

SUMMARY

Complete transection of the hypophyseal stalk in laying hens, caused an immediate and unregained loss of body weight, complete atrophy of the ovary, oviduct and comb, thus producing a condition closely resembling hypophysectomy as far as gonadal function is concerned.

Stalk section did not significantly alter secretion of either thyrotrophic or adrenotrophic hormones as judged by the normal histology and weight of their target glands, the molting pattern of hens or their ability to withstand sudden temperature changes and prolonged nocturnal fasts. All of these are drastically and negatively affected by hypophysectomy.

For these reasons the data presented are thought to support the theory that the rate of secretion and release of the gonadotrophic complex in birds is governed by a hypothalamic neurohumor via the portal system.

It seems unlikely that the partial hypofunction of the pituitary gland after stalk transection is due to ischemia. The latter should result in panhypopituitarism rather than in the decreased flow of only the gonadotrophic complex.

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SELECTIVE EFFECTS OF GROWTH HORMONE ON RATS FED A TRYPTOPHAN-LOW DIET[1]

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CONSIDERABLE data are available indicating that growth hormone administration will not promote a weight increment in animals that are nutritionally deficient. Thus growth-promoting extracts of the anterior pituitary failed to promote a gain in body weight in rats deficient in vitamin A (Margitay-Becht and Wallner, 1937; Ershoff and Deuel, 1945), thiamine (Simpson and Evans, 1944), the essential fatty acids (Deuel *et al.*, 1950), pantothenic acid (Lotspeich, 1950; Beare *et al.*, 1954), pyridoxine (Beare *et al.*, 1953), choline (Hall and Bieri, 1953) or riboflavin (Beare *et al.*, 1954). Similarly growth hormone failed to promote an increment in body weight in rats fed a low protein (6% casein) diet (Gordan *et al.*, 1947, 1948). Supplementing the above diet with dl-methionine or increasing the casein content to a minimum of 12% of the ration resulted, however, in a significant weight increment following growth hormone treatment. Chow and Greep (1948) have reported that the quality as well as the amount of dietary protein is also of importance in conditioning the response to growth hormone administration. These workers found that the gain in body weight of immature hypophysectomized rats treated with a growth-promoting fraction of the anterior ptiuitary was appreciably less when the source of dietary protein was soya protein than when it was casein or lactalbumin. In the present communication, data are presented on the effects of growth hormone administration on rats fed a tryptophan-deficient ration.

EXPERIMENTAL

Experiment #1. The effects of graded levels of dl-tryptophan on the weight increment of rats fed a tryptophan-free ration.

The basal tryptophan-free ration employed in the present experiment had the following composition: sucrose, 60.8%; acid hydrolyzed casein,[2] 24%; cottonseed oil (Wesson),

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[2] The acid hydrolyzed casein employed in this study was kindly provided by Dr. Harry Wax of the Stuart Company, Burbank, California.

10%; salt mixture,[3] 5%; and l-cystine, 0.2%. To each kg. of the above were added the following crystalline vitamins: thiamine hydrochloride, 40 mg.; riboflavin, 20 mg.; pyridoxine hydrochloride, 20 mg.; calcium pantothenate, 100 mg.; nicotinic acid, 100 mg.; ascorbic acid, 200 mg.; biotin, 4 mg.; folic acid, 10 mg.; para-aminobenzoic acid, 400 mg.; inositol, 800 mg.; vitamin, B_{12}, 150 micrograms; 2-methyl-napthoquinone, 5 mg.; choline cholride, 2 gm.; vitamin A, 5000 U.S.P., units; vitamin D_2, 500 U.S.P. units; and alpha tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. In addition to the basal ration, the following diets were also employed, consisting of basal ration plus each of the following supplements: (a) 600 mg. dl-tryptophan per kg. of diet, (b) 1.8 gm. dl-tryptophan per kg. of diet and (c) 5.4 gm. dl-tryptophan per kg. of diet. These supplements were incorporated in the diet in place of an equal amount of hydrolysate and corresponded to 0.25%, 0.75% and 2.25% respectively of the hydrolysate content of the basal ration. In addition to the above diets, tests were also conducted with a diet identical to the basal ration except that the acid hydrolysate was replaced by intact casein.[4]

Fifty albino female rats of the University of Southern California strain were selected at 21 to 24 days of age and an average body weight of 41 gm. (range 35 to 51 gm.) for the following experiment. Animals were placed in metal cages with raised screen bottoms and were fed the above diets *ad libitum* (10 rats per group). Diets were made up weekly and stored under refrigeration when not in use. Rats were fed daily, and all food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 28 days or until death, whichever occurred sooner. Rats fed the basal tryptophan-free ration lost weight from the start of the experiment and all animals in this group died during the fourth week of feeding. No deaths occurred on any of the other diets tested, but significant differences in weight increment were observed between rats in the various groups. After 28 days of feeding, the average weight increment of rats on the supplemented rations was as follows: basal ration plus 600 mg. dl-tryptophan per kg. of diet, 12.2 gm. (range 7–18 gm.); basal ration plus 1.8 gm. dl-tryptophan per kg. of diet, 47.5 gm. (range 37–59 gm.); basal ration plus 5.4 gm. dl-tryptophan per kg. of diet, 41.8 gm. (range 33–50 gm.); basal ration with intact casein, 99.8 gm. (range 76–111 gm.). Findings indicate that the addition of dl-tryptophan to the basal ration at a level of 600 mg. per kg. of diet was sufficient to keep the animals alive and to promote a slight increment in weight (12 gm. over a four week period). Higher levels of dl-tryptophan supplementation resulted in a marked increment in weight but growth was still significantly less than that obtained on the intact casein ration. These findings suggest that the acid hydrolysate employed in the present experiment may have been deficient not only in tryptophan but in some other factor or factors present in the intact casein.

Experiment #2. Effects of growth hormone administration on rats fed a tryptophan-low diet.

Twenty-four albino female rats of the University of Southern California strain were selected at 21 to 24 days of age and an average body weight of 43 gm. (range 38 to 50 gm.) and were fed *ad libitum* the basal tryptophan-free ration indicated above supplemented with 600 mg. dl-tryptophan per kg. of diet. After 28 days of feeding 20 of these rats (the 2 largest and 2 smallest in the group were discarded) were divided into 2 groups of 10 each and were treated as follows. One group received 10 daily intraperitoneal

[3] Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

injections of 0.5 ml. saline solution; the second group received 10 daily intraperitoneal injections of 0.5 ml. growth hormone[5] solution containing 100 micrograms of growth hormone. Animals were kept in individual metal cages with raised screen bottoms and were continued on the tryptophan-low ration. Food consumption and body weight were determined daily for each rat. Animals were autopsied 24 hours after the last injection. The heads and tibias were removed at the time of sacrifice and fixed immediately in an 80% alcoholic Bouin's (picric-acetic-formalin) solution for at least four days. The specimens were decalcified in 10% nitric acid in 10% formalin and washed with a saturated lithium carbonate solution. They were then dehydrated and infiltrated in the routine manner, embedded in 50% nitrocellulose, sectioned at 20–30 μ in thickness and stained with hematoxylin and triosin.

No significant differences in weight increment or food consumption were observed between the two groups. Rats receiving the saline injections gained an average of 5.1 ± 1.2 gm.[6] and those administered the growth hormone gained an average of 5.4 ± 1.3 gm. during the 10 day experimental period. The average food consumption per rat was 9.4 gm. per day for animals in the saline series and 9.7 gm. per day for rats in the growth hormone series. Findings indicate, therefore, that under conditions of the present experiment growth hormone failed to promote an increment in body weight in rats fed a tryptophan-low diet. Histologically, however, significant differences were observed between rats in the saline and growth hormone series. A marked narrowing of the epiphyseal plate was noted in the tibias of rats administered saline (Fig. 2). This decrease in the width of the cartilaginous layer was the result of a decrease in the number and size of the cells making up the various layers. There was also an indication of premature closure of the epiphyseal cartilage as characterized by discrete deposition of lamellar bone adjacent to the cartilage. In addition there was loss of bone trabeculae in both the epiphysis and diaphysis. The response of the epiphyseal plate of rats fed the tryptophan-low diet to the administration of growth hormone is illustrated in Figure 3. These animals exhibited an increase in chondrogenic activity in all the layers of the epiphyseal plate to a degree comparable to that of animals of the same age which had been fed a natural food stock ration (Fig. 1). The average width of the epiphyseal cartilage of rats of the groups represented by Figures 1 to 3 re-

[5] Beef STH (lot #728–074) kindly provided by Dr. Sanford L. Steelman of the Armour Laboratories, Chicago, Illinois. This material had an activity of 90–100% of Armour Standard. When assayed on hypophysectomized female rats, 15 to 20 mcg. of the above material per rat per day, according to Dr. Steelman, produced a gain of 1 to $1\frac{1}{2}$ gm. per day. The powdered hormone material was suspended in water and dilute alkali (NaOH) added to effect a solution. The volume was adjusted to a concentration of 200 mcg. per ml.

[6] Including standard error of the mean calculated as follows:

$$\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

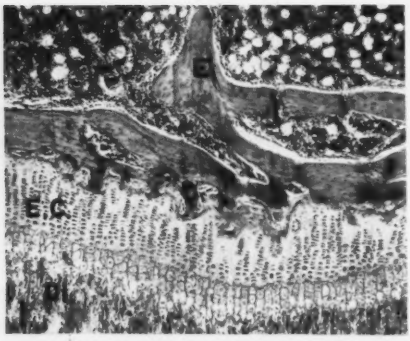

FIG. 1. Proximal epiphyseal region of the tibia of a rat fed a natural food stock ration. Note the width and the cellular morphology of the 4 layers of the epiphyseal cartilage (resting layer, proliferating layer, hypertrophied layer and calcifying layer). ×50.

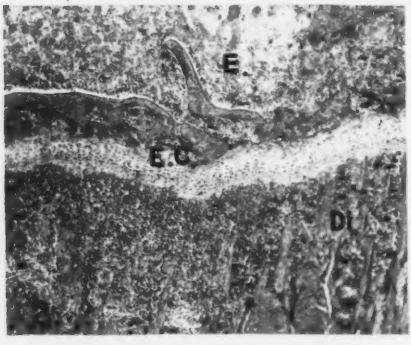

FIG. 2. Proximal epiphyseal region of the tibia of a saline injected rat fed a tryptophan-low diet. Note the narrow width of the epiphyseal cartilage and the concomitant reduction in the size and number of cells making up its 4 zones. Note also the beginning loss of trabeculation in the epiphysis and diaphysis. ×50.

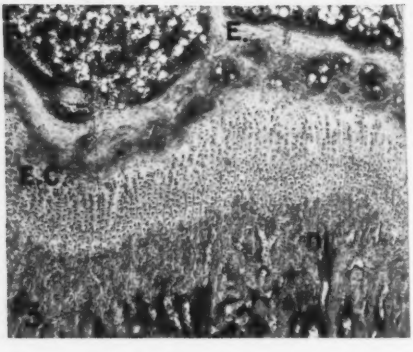

FIG. 3. Proximal epiphyseal region of the tibia of a rat administered growth hormone in conjunction with a tryptophan-low diet. Note the resumption of active chondrogenesis and osteogenesis as indicated by the increased number of cells in each of the 4 zones. Note also the appearance of trabeculation in the diaphysis and epiphysis. ×50.

KEY TO ABBREVIATIONS

Dd.	Deficient dentin	Ir.	Interradicular area of molar tooth
Di.	Diaphysis	M.	Marrow space
E.	Epiphysis	O.	Osteoblastic layer
E.C.	Epiphyseal cartilage	Od.	Odontoblastic layer
G.	Gingiva	P.	Pulp
Hd.	Growth Hormone dentin	T.	Trabecula

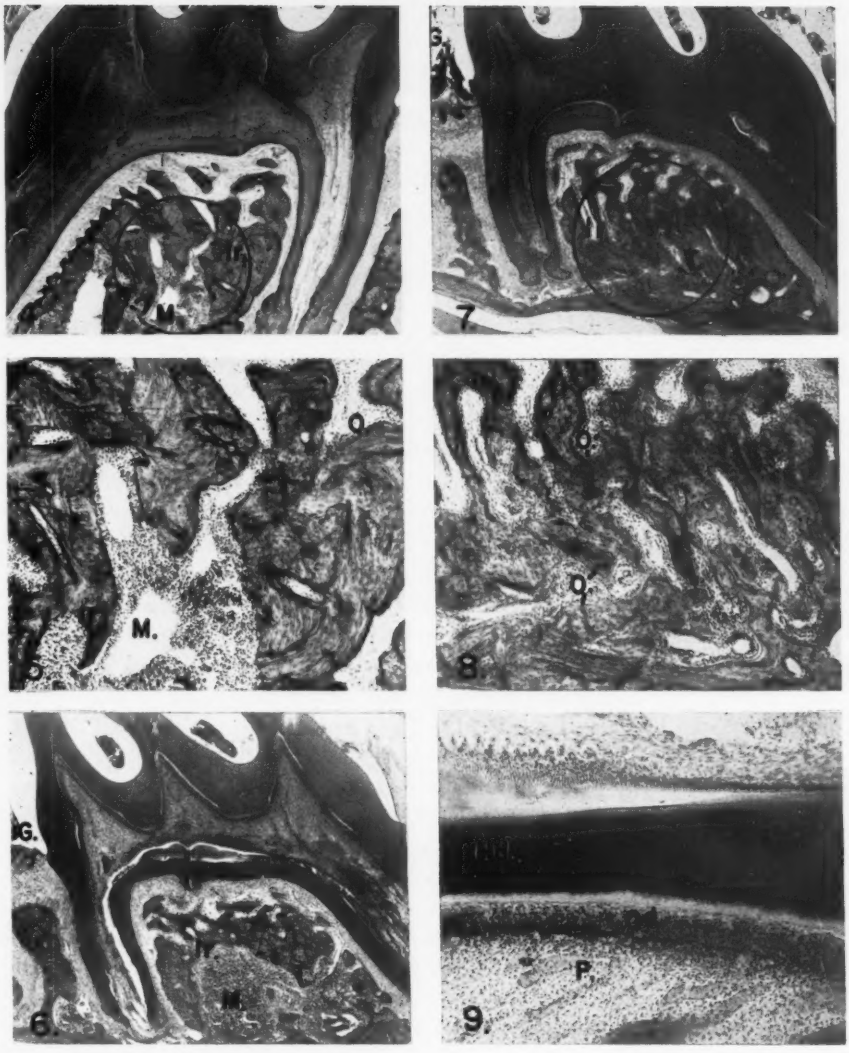

FIG. 4. Upper first molar region of a rat fed a natural food stock ration. Note the wide trabeculae in the interradicular area with marrow spaces filled with myeloid tissue. ×50.

FIG. 5. Higher magnification of the encircled area of Fig. 4. Note the trabeculae separated by the marrow spaces filled with myeloid tissue. There is very little osteoblastic activity present. ×100.

FIG. 6. Upper first molar region of a saline treated rat fed a tryptophan-low diet. Note the osteoporosis in the interradicular bone. ×50.

FIG. 7. Upper first molar region of a rat administered growth hormone in conjunction with a tryptophan-low diet. Note the dense deposition of bone in the interradicular area. ×50.

FIG. 8. Higher magnification of the encircled area of Fig. 7. Note the trabeculae and decrease in the size of the marrow spaces. The latter are filled with connective tissue in contrast to the myeloid tissue shown in Fig. 5. ×100.

FIG. 9. Incisal dentin from a rat administered growth hormone in conjunction with a tryptophan-low diet. The pre-hormonal dentin (Dd.) shows an increased number of interglobular spaces, whereas the dentin deposited after the administration of growth hormone (Hd.) appears homogeneous. ×50.

spectively was as follows: Natural food stock ration (Fig. 1), 252 μ; tryptophan-low diet plus saline injections (Fig. 2), 148 micra; and tryptophan-low diet plus growth hormone injections (Fig. 3), 259 micra. Although no difference was observed in the width of the epiphyseal plate between hormone treated rats and the natural food stock controls, there was less intercellular material in the hormone treated animals, a finding indicative of a more active chondrogenesis in the hormone treated series.

Differences in the periodontium of molar teeth were also noted between rats in the saline and growth hormone series. Osteoporosis of the spongiosa of the interradicular region was present in all rats of the saline group (Fig. 6). The cribriform plate and the adjacent periodontal membrane fibers however, remained intact. In contrast, rats in the hormone treated group exhibited bone deposition in the spongiosa of the interradicular bone which largely obliterated the marrow spaces (Fig. 7 and 8). The appearance of the interradicular region of rats of a similar age but fed a natural food stock ration is illustrated in Figures 4 and 5. The trabeculae of these animals were well developed with marrow spaces filled with myeloid tissue. Very little osteoblastic activity was noted in these rats in contrast to the picture presented by the hormone treated animals. The interproximal gingiva of rats in both the saline and hormone treated groups showed ulceration of the superficial layer and its basal cellular region had proliferated sending rete pegs into the underlying connective tissue which was infiltrated with inflammatory cells. These findings, however, were observed to an equal extent in both the saline and growth hormone series. The incisal dentin of saline treated rats on the tryptophan-low diet was characterized by an increased number of interglobular spaces. The dentin laid down during the period of growth hormone administration, however, appeared homogeneous in structure. The effects of growth hormone administration on the incisal dentin are demonstrated in Figure 9 which illustrates both the poorly calcified dentin deposited during the period prior to hormone administration and the homogeneous appearing dentin laid down subsequently.

DISCUSSION

Present findings indicate that rats fed a tryptophan-low diet failed to respond to injected growth hormone by an increment in body weight but that such treatment did result in a stimulation in bone growth. These findings suggest either (1) that chondrogenesis and osteogenesis may be more sensitive than increment in body weight as an index of growth hormone activity or (2) that a dietary deficiency of tryptophan might interfere with one manifestation of growth hormone activity (i.e., increment in body weight) whereas another might remain relatively unimpaired. In accord with the first of these possibilities, Denko and Bergenstal (1955) have presented data indicating that increased S^{35} fixation in the cartilage

of hypophysectomized rats is a more sensitive indicator of growth hormone activity than increment in body weight. In accord with the latter possibility, however, Beare *et al.* (1954) found that rats deprived of pantothenic acid gained less weight following growth hormone administration than rats on a control diet although nitrogen-retention was unimpaired.

SUMMARY

Administration of growth hormone failed to promote an increment in body weight in rats fed a tryptophan-low diet. Growth hormone therapy, however, did cause a resumption of chondrogenesis and osteogenesis in the epiphyseal region of the tibia, bone formation in alveolar bone and the formation of normal appearing incisal dentin.

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CONVERSION OF STEROIDS TO ALDOSTERONE-LIKE MATERIAL[1]

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WETTSTEIN and co-workers (1955) described the formation of material possessing the chromatographic and biological properties of aldosterone by incubation of desoxycorticosterone with adrenal homogenates. In addition, it was shown that corticosterone, progesterone and pregnenolone decreased the yield of aldosterone under the same conditions. Since we have been able to demonstrate the presence of an aldosterone-like material in the effluent of isolated calf adrenal glands perfused with a synthetic perfusion medium (Ungar *et al.*, 1954), this technique has been employed to study the bioconversion of possible steroid precursors to aldosterone. The results obtained by the perfusion of three such steroids, viz., corticosterone, desoxycorticosterone, and progesterone, form the subject matter of this communication.

METHODS AND MATERIALS

Desoxycorticosterone, corticosterone, and progesterone in 10 mg. amounts were dissolved in 0.5 ml. of propylene glycol and were added individually to one liter of perfusion medium consisting of a glucose-fortified, physiologically balanced and buffered salt solution (Rosenfeld, 1955). Each steroid solution was perfused for a period of 2 hours at 37.5° C in a multicycle system through four calf adrenals. The four contralateral glands, perfused concurrently in a second system without added steroid, served as the control. The effluents were frozen upon collection in a dry ice-acetone mixture and kept in this state until extraction was undertaken. Each thawed perfusate was extracted four times with fresh portions of 1/3 volumes of redistilled ethyl acetate. The ethyl acetate extract was treated with sodium sulfate and evaporated under reduced pressure. The residue was then chromatographed on paper for 72 hours in the toluene-propylene glycol system (Zaffaroni *et al.*, 1950). The zone which migrated in front of cortisol and contained cor-

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[2] The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

tisone plus aldosterone was eluted and bioassayed for sodium-retaining activity. In some experiments, this eluted zone was rechromatographed in a benzene-ethyl acetate-methanol-water system (Bush, 1952) in order to separate the cortisone and aldosterone-like components. The zone which would contain aldosterone was then eluted and analyzed by bioassay.

The bioassay procedure consisted of a modification of that described by Marcus *et al.* (1952), and employed the bilaterally-adrenalectomized male rat weighing 135–150 g. Each animal was injected intraperitoneally with 45 mg. of sodium chloride and urine was collected for 6 hours. Urinary sodium was determined with a Perkin Elmer Flame Photometer, Model XX, using an internal lithium standard. The experimental and control perfusate materials were bioassayed simultaneously with a solvent control and two standards of desoxycorticosterone. The 8 animals employed in the bioassay of each unknown, solvent control and standard were grouped in pairs. Either two or three independent bioassays were conducted for each conversion study.

RESULTS

The perfusion of corticosterone through the isolated calf adrenal produced a small increment in aldosterone-like material (Table 1). The rats

TABLE 1. PERFUSION OF 10 MG. CORTICOSTERONE AND PRODUCTION OF SODIUM-RETAINING MATERIAL

Experiment No.	Aliquot of control and corticosterone perfusate extracts* administered per rat	Mean urinary sodium excretion mg./100 gm./hour (Range)				
		Solvent control	Desoxycorticosterone standard		Control perfusate	Corticosterone perfusate
			6 μg.	25 μg.		
1	1/30	2.13 (1.50–2.80)	1.37 (1.31–1.47)	1.20 (1.20–1.41)	1.57 (1.18–2.22)	1.32 (1.13–1.63)
2	1/60	1.78 (1.45–2.06)	1.31 (0.80–1.60)	0.89 (0.69–1.32)	2.17 (1.35–2.60)	1.79 (1.64–1.96)
3	1/30	1.69 (1.42–1.88)	1.26 (0.96–1.61)	0.87 (0.67–1.14)	1.33 (1.09–1.72)	1.10 (0.86–1.31)

* Zone containing aldosterone and cortisone eluted after chromatographic separation in Zaffaroni system.

of two bioassays injected with 1/30th of the total control extract showed a mean sodium excretion of 1.45 mg. per 100 gm. body weight per hour as compared to 1.21 for those animals which received 1/30th the material derived from the corticosterone perfusion. At the 1/60th dosage level, the mean urinary Na excretion of the animals treated with the experimental extract was not significantly different from that of the solvent controls (1.79 and 1.78 mg./100 gm./hour, respectively). In the same perfusion, the conversion of corticosterone to cortisol and cortisone was demonstrated by a twofold increase of these components over the control values.

The 2-hour multicycle perfusion of 10 mg. of desoxycorticosterone through the isolated calf adrenal resulted in a 30% conversion to corticosterone but no significant increase in aldosterone production. The so-

TABLE 2. PERFUSION OF 10 MG. DESOXYCORTICOSTERONE AND PRODUCTION OF SODIUM-RETAINING MATERIAL

Experiment No.	Aliquot of control and desoxycorticosterone perfusate extracts* administered per rat	Mean urinary sodium excretion mg./100 gm./hour (Range)				
		Solvent control	Desoxycorticosterone standard		Control perfusate	Desoxycorticosterone perfusate
			6 μg.	25 μg.		
4	1/30	2.13 (1.50–2.80)	1.37 (1.31–1.47)	1.20 (1.02–1.41)	1.46 (1.07–1.88)	1.75 (1.09–2.21)
5	1/15	1.69 (1.42–1.88)	1.26 (0.96–1.61)	0.87 (0.67–1.14)	1.38 (0.86–1.72)	1.80 (1.52–2.30)

* Zone containing aldosterone and cortisone eluted after chromatographic separation in Zaffaroni system.

dium excretion values for the desoxycorticosterone experimental perfusate were actually greater than those for the control perfusate at the 1/30th and 1/15th bioassay dosage levels (Table 2).

In contrast to corticosterone and desoxycorticosterone, an equal amount of progesterone, similarly perfused through calf adrenal glands, resulted in a significantly augmented output of aldosterone-like material (Table 3). At a dosage level of 1/30th of the experimental and control extracts, the sodium excretion decreased significantly from the solvent control value of 2.13 mg./100 gm./hr. to 1.32 for the control perfusate, and 0.89 for the progesterone perfusate. These extracts were re-assayed twice at a 1/60th dosage level. The combined data showed a mean sodium excretion value of 1.89 ± 0.17 mg./100 gm./hr. for the control solvent, 1.45 ± 0.15 for the control perfusate material, and 0.95 ± 0.09 for the extract obtained from the progesterone perfusion. The t value for the difference in the latter two was found to be 2.941 giving a probability value P of less than 0.05.

TABLE 3. PERFUSION OF 10 MG. PROGESTERONE AND PRODUCTION OF SODIUM-RETAINING MATERIAL

Experiment No.	Aliquot of control and progesterone perfusate extracts* administered per rat	Mean urinary sodium excretion mg./100 gm./hour (Range)				
		Solvent control	Desoxycorticosterone standard		Control perfusate	Experimental perfusate
			6 μg.	25 μg.		
6	1/30	2.13 (1.50–2.80)	1.37 (1.31–1.47)	1.20 (1.02–1.41)	1.32 (1.05–1.88)	0.89 (0.78–1.10)
7	1/60	1.81 (1.72–1.92)	1.31 (0.77–1.81)	0.84 (0.55–1.07)	1.35 (0.85–1.96)	1.01 (0.89–1.12)
8	1/60	1.98 (1.70–2.41)	1.27 (0.90–1.51)	0.89 (0.63–1.14)	1.55 (1.14–2.09)	0.88 (0.43–1.27)

* Aldosterone zone eluted after chromatographic separation in Zaffaroni and Bush systems.

DISCUSSION

The results of perfusing progesterone in an artificial medium through the isolated calf adrenal demonstrated that this steroid can serve as a precusor of aldosterone-like material. Under the same experimental conditions, corticosterone and desoxycorticosterone were not significantly converted. Corticosterone caused only a small increment in the Na-retaining activity of the calf adrenal effluent whereas the desoxycorticosterone effluent showed even less aldosterone-like material than the control. These findings are at variance with those obtained by incubation of the same steroids with beef adrenal homogenates, as reported by Wettstein *et al.* (1955). Although a significant conversion to aldosterone did not occur, the perfusion of corticosterone resulted in a two-fold increase in cortisone and cortisol, while desoxycorticosterone was converted similarly to corticosterone in a 30% yield. These metabolic transformations suggest that permeability factors alone could not explain the differences seen in the perfusion and homogenate studies in regard to steroid precursor and aldosterone relationships. The differences observed may be due to the inhibitory (perhaps nonspecific) effects exerted by relatively large amounts of steroid in the homogenate system. In addition, as noted by these authors, the course of the reactions may be altered under the conditions employed.

Since desoxycorticosterone was not effective as a precursor, and corticosterone produced at best only small quantities of aldosterone, it would appear that 18-hydroxylation and/or the formation of 18-aldehyde from the 18-methyl group is more easily carried out before 11β- and 21-hydroxylations have occurred. Kahnt, Neher and Wettstein (1956) have incubated desoxycorticosterone-21-C^{14} with beef adrenal homogenates and have isolated aldosterone labeled exclusively in carbon 21. The radioactivity in the aldosterone formed by direct conversion, however, accounted for only 48 per cent of the theoretical total, suggesting that other natural precursors are present in the adrenal. In addition, these authors (Kahnt *et al.*, 1955) have demonstrated the 18-hydroxylation of desoxycorticosterone by beef adrenal homogenates. The role of this compound as an intermediate in the biosynthesis of aldosterone has not yet been determined.

On a theoretical basis, the route from progesterone to aldosterone may take many different pathways. Desoxycorticosterone is a relatively direct precursor of aldosterone on the basis of the results of Kahnt, Neher and Wettstein (1956). On the basis of our data and under the conditions employed, it appears that desoxycorticosterone and corticosterone are less efficient precursors of aldosterone suggesting an alternative pathway. Progesterone (I), as a possible intermediate, may be converted to 18-hydroxyprogesterone (III), which following 21- and 11β-hydroxylations would in turn be converted to aldosterone (XII) (Fig. 1). Another alternative would be the conversion of progesterone (I) to 11β-hydroxyprogesterone (VII), which upon hydroxylation at C-21 and C-18 would form aldosterone (XII).

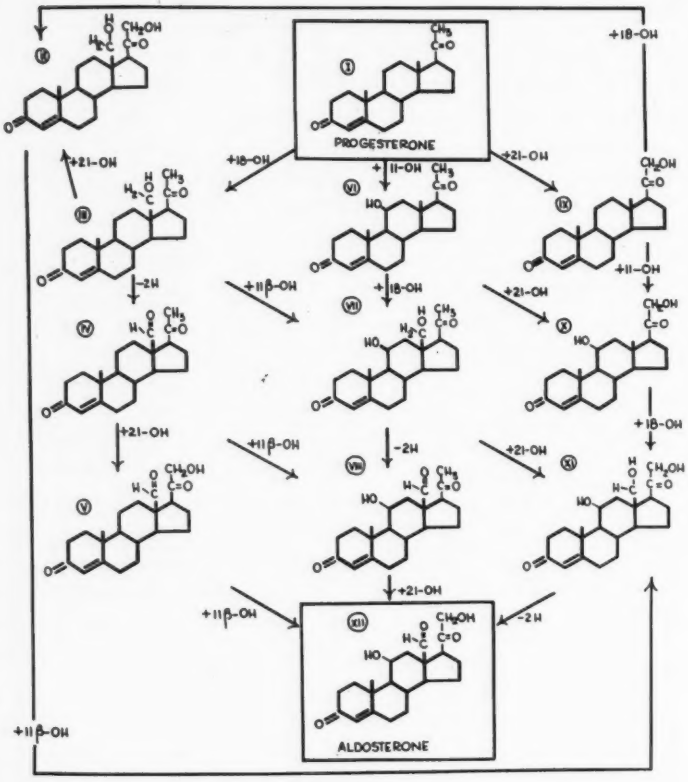

FIG. 1. Possible routes for the biosynthesis of aldosterone (XII) from progesterone (I).

Further perfusion experiments with 11β-hydroxyprogesterone (VII), 18-hydroxyprogesterone (III), and 18-hydroxydesoxycorticosterone (II) as possible intermediates are indicated to elucidate the metabolic pathway of aldosterone production.

SUMMARY

Desoxycorticosterone, corticosterone and progesterone were added to a synthetic medium and perfused through the isolated calf adrenal gland to assess their roles as possible intermediates in the biosynthesis of aldosterone. The effluents were fractionated by paper chromatography and their content of aldosterone-like material determined by bioassay. On the basis of the present findings and under the conditions employed, desoxycorticosterone did not qualify as a precursor of aldosterone, corticosterone gave equivocal results, while progesterone perfusion resulted in a statistically significant increase in the production of aldosterone-like material.

Acknowledgment

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THE STRUCTURE OF THE HUMAN ADRENAL CORTEX[1,2]

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THERE is no other organ which shows greater specific differences in structure than the adrenal cortex. Observations of recent date (Gruenwald, 1942; Gruenwald and Konikov, 1944 and Elias, 1948) have shown that these differences are so striking that identification of a species on the basis of its adrenal histology is very easy. The object of this paper is to attempt to clarify the architecture of the adrenal cortex in our own species. Knowledge of the structure of our own adrenal cortex is especially important, because we must know in which way we differ from those animals which are used for experimentation.

A historical account on work done is not necessary since this gigantic task has been masterfully accomplished by Bachmann (1954). References to the literature will be cited at the appropriate places under "observations."

MATERIAL AND METHODS

The study was begun using adrenal glands obtained during 20 routine necropsies at the Department of Pathology of Cook County Hospital. For this material we are indebted to Dr. Hans Popper. This material was used to gain some preliminary ideas on how the work could be organized. There existed a great variety of structures not only from case to case but also within every individual gland. The material was found to lack the constancy desirable for generalizing statements. Could the variability of structure perhaps be attributed to the various diseases of which the patients had died? It was impossible to tell. It was therefore decided to examine material of healthy persons only.

Consequently 28 adrenal bodies of 14 healthy persons who suffered sudden, unexpected, accidental death were obtained from the office of the coroner of Cook County, Illinois. Also, in this material a great variety of structures was found. The accident victims of this second series ranged in age from 15 to 63 years and included both sexes.

In an attempt to obtain a more uniform material we obtained, subsequently, permission to study the glands of each of 19 accidental electrocution cases of service men who died on active duty. These cases were made available through the courtesy of the Armed Forces Institute of Pathology. Observing slides of the other organs of these cases demonstrated that the physical act of electrocution had produced no noticeable microanatomical changes. It was therefore assumed that this material could be considered as normal. The accidental electrocution cases were, then, compared with the other ac-

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[1] Aided by a grant from the U. S. Public Health Service.

[2] Presented at the 102nd annual session of the American Medical Association, New York City, 1953.

cidental death cases; and it was found that they did not differ in any noticeable form from them. Retrospectively, then, one could assume that also the material from various other kinds of accidental deaths (falls, automobile accidents, etc.) could be considered normal.

Further, 9 cases of suicide by hanging, 5 cases of suicide by gun shot wounds, one case of judicial electrocution and one case of judicial hanging were studied at the Armed Forces Institute of Pathology. Also these cases of physically healthy persons who died after considerable psychological stress were not found different, in essence, from the other groups.

It was stated above that the variations of structure from individual to individual, between left and right and between various areas of the same gland are striking. On the other hand it was stated that the various groups of material examined were uniform. These appear to be contradictory statements. However, if groups are found to resemble each other so that they cannot be distinguished, we are dealing with a statistically balanced material. Yes, each adrenal cortex is strikingly different from those of other individuals; and it may sometimes be very much different from its contralateral partner. Yet the same types of structural elements occur in a statistically significant distribution in each group; and this distribution of structural types is very much the same in all groups examined.

Since the adrenal cortex differs so much from other organs in its variability of structure, an unconventionally large portion of space had to be given to the problem of selection and evaluation of cases.

The glands of all accident cases used in this study were fixed in 10% Formalin within $3\frac{1}{2}$ hours after death. The glands from routine necropsies and from the sudden, unexpected death cases were cut into serial sections of 10μ thickness. The majority of slides was routinely treated with Masson's trichrome stain. One slide of each case was stained with hematoxylin and eosin, several slides with van Gieson's picrofuchsin and one to five slides were impregnated with silver after the method of Gomori.

To determine the distribution of lipoids, cholesterol esters and steroids, frozen sections were stained with sudan IV and others were exposed to the Schultz reaction test.

Three dimensional reconstructions were prepared by projecting serial sections with a table microprojector on lantern slide cover glasses as described by Elias (1955). The images were traced on these glass plates with opaque inks of various colors and partially painted with a transparent, colored acetate lacquer (pink nail polish). These serial glass plates were stacked, and stereograms were drawn from them.

For the analysis of the connective tissue structure, the statistico-geometrical method of Elias and Spanier (1953) was used.

OBSERVATIONS

Stroma

1. *The zones as defined by the arrangement of argyrophil tissue*

Ever since Arnold (1866) wrote his classical paper, we have been distinguishing three zones of the adrenal cortex to which must be added, as an integral part, the capsule. In the modern literature as well as in textbooks, there reigns confusion concerning the identification of the zones. It will therefore be necessary to return to the original description by Arnold which is largely based on the connective tissue and its relation to the parenchyma in cattle and man.

Arnold describes the cortex roughly as follows: From the compact capsule, there

project inward into the cortex strands and sheets of connective tissue at oblique angles. Beneath the capsule, these connective tissue tracts include rather large "caves" (Höhlen) which are filled with "parenchymal bodies" (cells). He calls the zone which contains these large parenchyma filled caves the zona glomerulosa. The connective tissue which forms the walls of the caves fringes out at a lower level to form straight, parallel tracts, more closely set than the septa of the zona glomerulosa and laterally connected with each other. Connective tissue fibers connect these vertical strands and form basketworks so fine that they surround individual, nucleated "parenchymal bodies" (cells). These lie, however, close together, being separated from each other only by thin networks of fibers. This arrangement prevails in the zona fasciculata. The deepest zone is characterized by still finer connective tissue fibers forming a dense feltwork. In this are suspended smaller "parenchymal bodies," spherical or elliptical, often at some distance from each other. The zone which has this arrangement is the zona reticularis.

We began our account with Arnold's description, because it was he to whom we owe the nomenclature of the zones. He worked with methods which were rather primitive even for his time. A much better account of the connective tissue of the adrenal cortex had been given 17 years earlier by Gerlach, whom we will quote verbatim:

"The capsule which surrounds the adrenal gland consists of connective tissue. The fiber bundles run parallel to the longitudinal diameter of the organ. From these bundles fiber tracts penetrate vertically into the cortical substance at distances of one-fourth to one-half lines of each other. The cortical substance is divided in this way into compartments of various sizes. The fiber tracts run through the entire cortical substance and dissipate before having reached the medulla." (translated)

Gerlach then explains that membranous sleeves are formed from these fiber tracts and continues: "These sleeves are formed by structureless membranes and possess a dense content which is chiefly formed of numerous elementary bodies (cells) and fat droplets."

We have translated the German work "Schlauch" with the English word "sleeve." The word "Drüsenschläuche" had been used already by Ecker in 1846. It was interpreted by later reviewers as "glandular tubules." For, indeed, the word is used in this sense in modern German histological nomenclature. It has therefore been said (Bachmann, 1954) that Ecker had described lumina in the cortical cell cords. However, it is obvious that the "Schläuche"[3] described by Ecker and Gerlach are sleeves, of connective tissue.

These sleeves are, according to Ecker and Gerlach, filled with parenchymal cells. It will be seen below that this concept corresponds exactly to our own observations.

Later 19th century authors, as Flint (1899), for example, have added little to the knowledge gained by Ecker, Gerlach and Arnold. But they all agree on the definition of zones based on connective tissue as established by Arnold.

A very thorough account of the argyrophil tissue was given by Comolli (1908). Also Gruenwald (1942) and Gruenwald and Konikov (1944) used a silver impregnation method to demonstrate reticular fibers. Their results are identical with those of Gerlach (1848), Arnold (1866) and Comolli (1908) but are much more precise and much more inclusive. The illustrations of Gruenwald and Konikov show a very characteristic pattern of reticular fibers in the guinea pig, cat, dog, rhesus monkey and cattle. Our Figures 17, 18, 19, 20 and 21 demonstrate this arrangement. The rat makes

[3] The German word *Schlauch* means a hose with thin flabby walls. Glandular tubules should rather be called "*Drüsenröhren*." For this word denotes a tube or pipe with a thick wall.

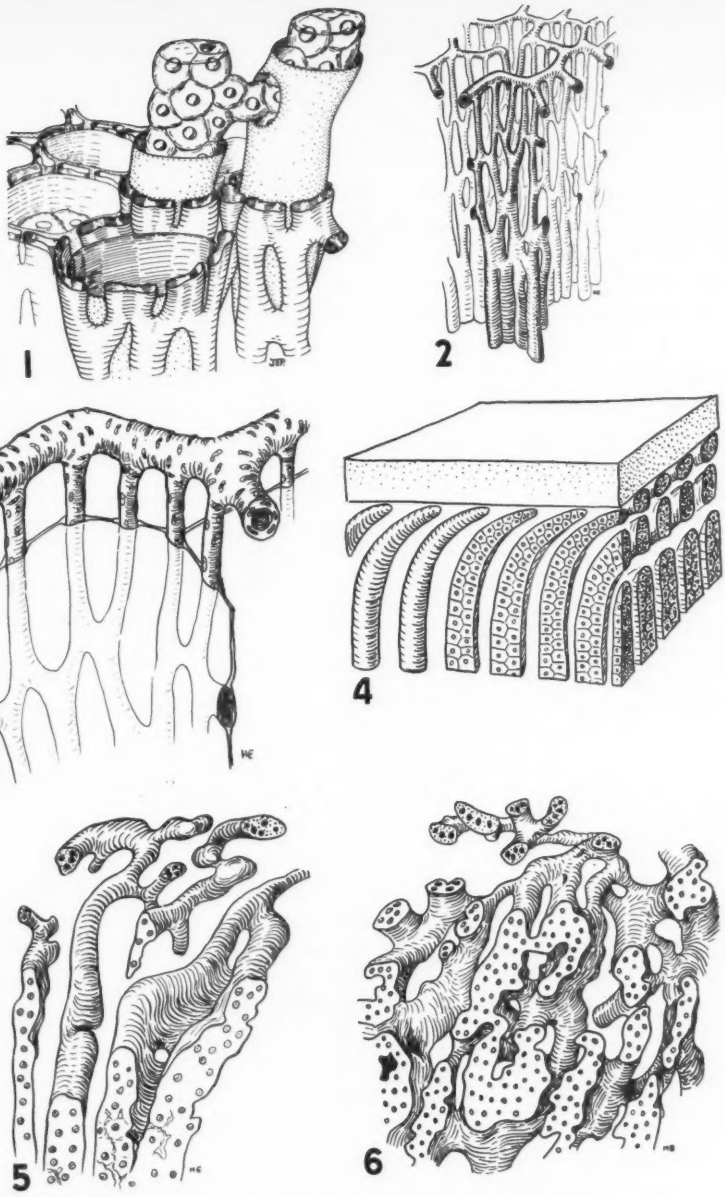

FIG. 1. *Stereogram** of the zona fasciculata.

FIG. 2. Stereogram of the vasculature of the capsule, zona glomerulosa and zona fasciculata.

FIG. 3. Stereogram showing the relationship of the capillaries to the subcapsular arterial plexus and to the membranous septa of the zona glomerulosa.

FIG. 4. Stereogram of typus levis zonae glomerulosae showing the effect of different directions of sectioning.

FIG. 5. *Reconstruction*† of typus levis connected (intracapsular blastema stippled).

FIG. 6. Reconstruction of typus nodosus (blastema stippled).

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* With the word *"Stereogram"* we mean a semi-schematical, three-dimensional presentation based on the observation of many specimens by various methods.

† We designate with the word *"Reconstruction"* an accurate three-dimension presentation of a specific area from a specific specimen based on serial sections.

an exception to the usual pattern (Pauly, 1954) having no zone in which not every individual cell is surrounded by reticular fibers (Fig. 22). But even in the rat the zona glomerulosa can be distinguished from the fasciculata by reticular fiber distribution. In the glomerulosa of the rat, the individual cell may be touched by one or two reticular fibers while in the fasciculata a great many fibers surround each cell in basketwork fashion.

In general it can be stated: The capsule is the dense layer of connective tissue located superficial to the zona glomerulosa. The zona glomerulosa is that stratum in which relatively large compartments exist which harbor large masses of cells. The compartments are separated from each other by intervening connective tissue septa. The zona fasciculata is that layer in which little baskets of reticular fibers surround individual cells. This layer is deep to the zona glomerulosa. The zone deep to the zona fasciculata is the zona reticularis. Its stroma is rather variable in structure but is noticeably denser than that of the outer two zones.

The zones of the adrenal cortex can therefore be identified with precision in silver impregnated slides demonstrating reticular fibers.

2. *The zones as defined by the fuchsinophil tissue*

If one stains sections of the adrenals with van Gieson's picrofuchsin a pattern is revealed which is slightly different from the argyrophilic pattern. It is obvious then that more than one kind of connective tissue exists in this organ and that these various kinds of tissue must be intermingled with each other.

Capsule. In van Gieson stained vertical sections, the capsule shows numerous red dots, many short lines and very few long lines (Fig. 23). A count of these figures undertaken on three randomly selected specimens sectioned in two directions perpendicular to each other, but both perpendicular to the surface of the gland, all sectioned at 10 μ thickness shows:

79.7% "dots," that is, little red specks or "commas" 20 μ or shorter.
18.8% "short lines," 21–40 μ long
1.1% "rather long lines," 41–80 μ long and
0.4% "very long lines" 81 μ or longer

These dots, commas and lines are the projections into the cutting plane of portions of fibers cut transversely, obliquely and longitudinally. The percentagewise distribution of these projections demonstrates the fibrous nature of the capsule, since it corresponds rather closely to the theoretically predicted distribution as explained by Elias and Spainer (1953).[4] "Fibers" are threads of practically infinite length and of approximately circular cross section with a diameter of negligible dimension. If such fibers were mathematical lines cut by a mathematical plane, infinitely thin, they all should appear as points. Since a histological "section" is

[4] 0–20μ: 86.2%; 20–40μ: 9.3%; 40–80μ: 3.3%; >80μ: 1.2%.

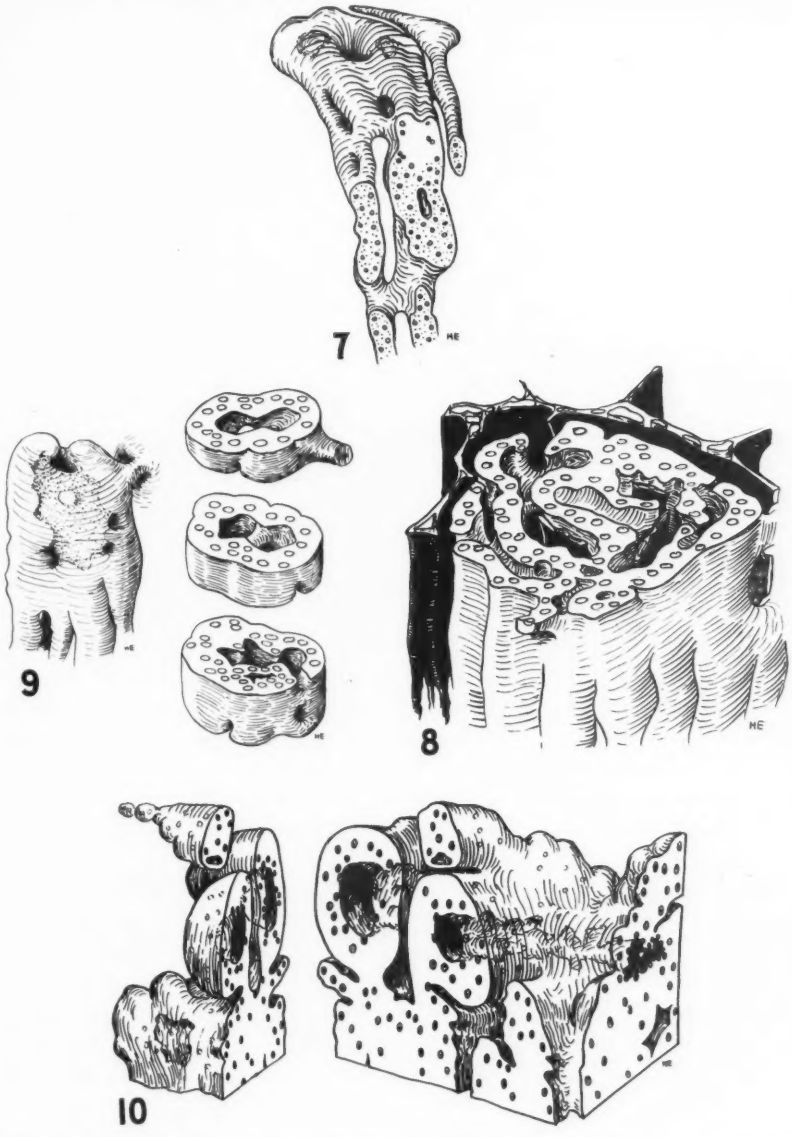


FIG. 7. Reconstruction of typus lipoidus (a case of syncytial arrangement). Notice the 2 lumina and the connection with blastema.

FIG. 8. Reconstruction of typus aperto-follicularis bovinus and of vascularized, membranous septa.

FIG. 9. Reconstruction of a small follicle of typus aperto-follicularis bovinus seen in its entirety (left) and cut into 3 slices, the upper one inverted (right).

FIG. 10. Reconstruction of two kinds of typus clauso-follicularis.

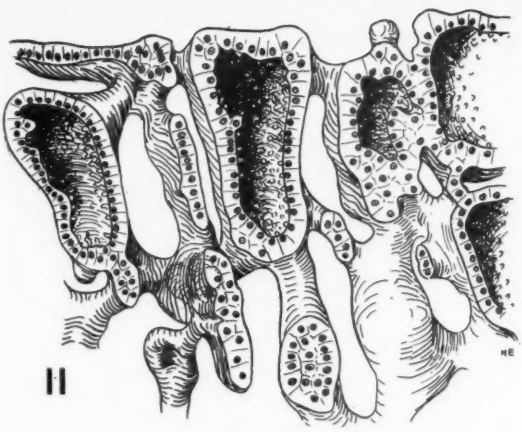
really a slice of considerable thickness, a portion of each fiber is included in the slice. This portion is seen from a direction perpendicular to the cutting plane. What we see of this fiber portion is really its projection into the cutting plane, $p = t/\tan \alpha$, where t is the thickness of the "section" and α the angle which the fiber forms with the cutting plane. The statistical theory of Elias and Spanier is based on this simple relation. In cross sections of the adrenal gland (Fig. 23) one can recognize that the fibers are oriented parallel to the surface of the organ. But a thick, tangential section shows these fibers running in all directions, crossing each other (Fig. 24). This very crossing of lines is another indication of the fibrous (thread like) nature of the connective tissue of the capsule; for only linear structures can cross in a portion of three dimensional space.

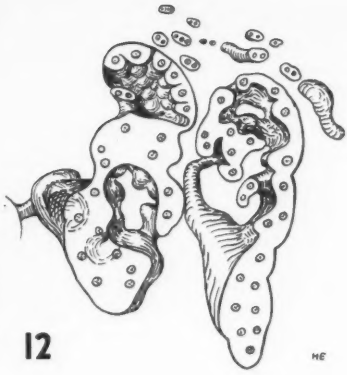
Zona glomerulosa: The fuchsinophil tissue of the zona glomerulosa shows an entirely different appearance in sections; for in this zone we find, in van Gieson stained slides, delicate long red lines, both in cross and tangential sections of the cortex. Dots or commas are rare. It follows that collagen fibers do almost not exist in the glomerulosa. Structures, the sections of which appear as lines, are membranes (Elias and Spanier, 1953; Elias and Pauly, 1954).

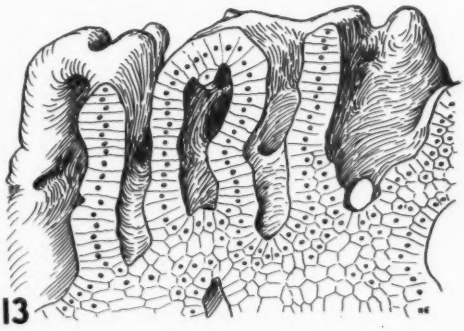
The membranes of the zona glomerulosa do not consist of collagen as recent, unpublished studies by Gershbein and Elias indicate. Both collagen fibers and membranes are digestible in pepsin; but while the membranes are digestible in trypsin, the collagen fibers are not. The substance of which the membranes consist is a protein different from elastin, reticulin and collagen as shown by staining and digestion reactions. It may be called *membranin* ($\mu\varepsilon\mu\beta\acute{\alpha}\nu\alpha$ = parchment). This word is not a chemical definition. But the term can be used with the same justification as the words "elastin" and "reticulin" have been coined on merely mechanical and morphological grounds.

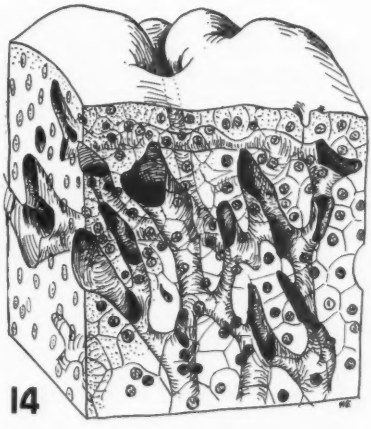
There is a third respect in which the membranes are different from the collagen fibers, and that is, that very few fibroblasts can be found in membranous connective tissue. This is not only so in the zona glomerulosa of the adrenal cortex but also in the membranous tissue of the cirrhotic, human liver (Elias and Spanier) and in the stroma of senile, human breasts, both female and male. (Observation soon to be published by Dr. Gershbein and one of the present writers.) This observation points to a genesis of these membranes different from that of collagen fibers.

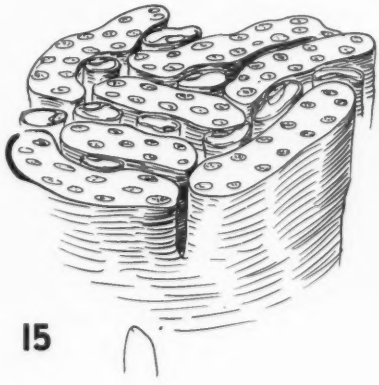
Zona fasciculata: At the boundary between the zonae glomerulosa and fasciculata, the membranous septa fringe out to form narrower and more delicate bands which are connected with each other. Unlike the argyrophil fibers, the membranous material does not penetrate between individual cells. Thus, the parenchymal cords of the fasciculata are ensheathed in thin walled, membranous tubes (Schläuche, Ecker). Neighboring tubes are connected with each other as the cells in honeycombs (Fig. 1).











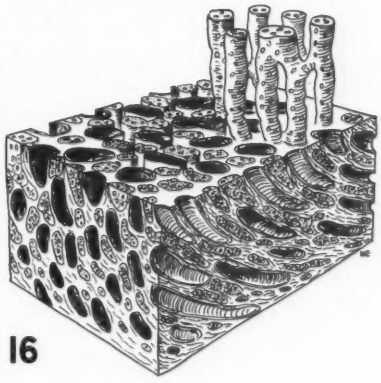


FIG. 11. Reconstruction of two kinds of typus clauso-follicularis.

FIG. 12. Reconstruction of typus aperto-follicularis atrophicus. Also intracapsular blastema (above).

FIG. 13. Reconstruction of typus arcuatus bovinus.

FIG. 14. Reconstruction of typus muraliosus.

FIG. 15. Stereogram of the rare plate type of zona fasciculata.

FIG. 16. Stereogram of zona reticularis.

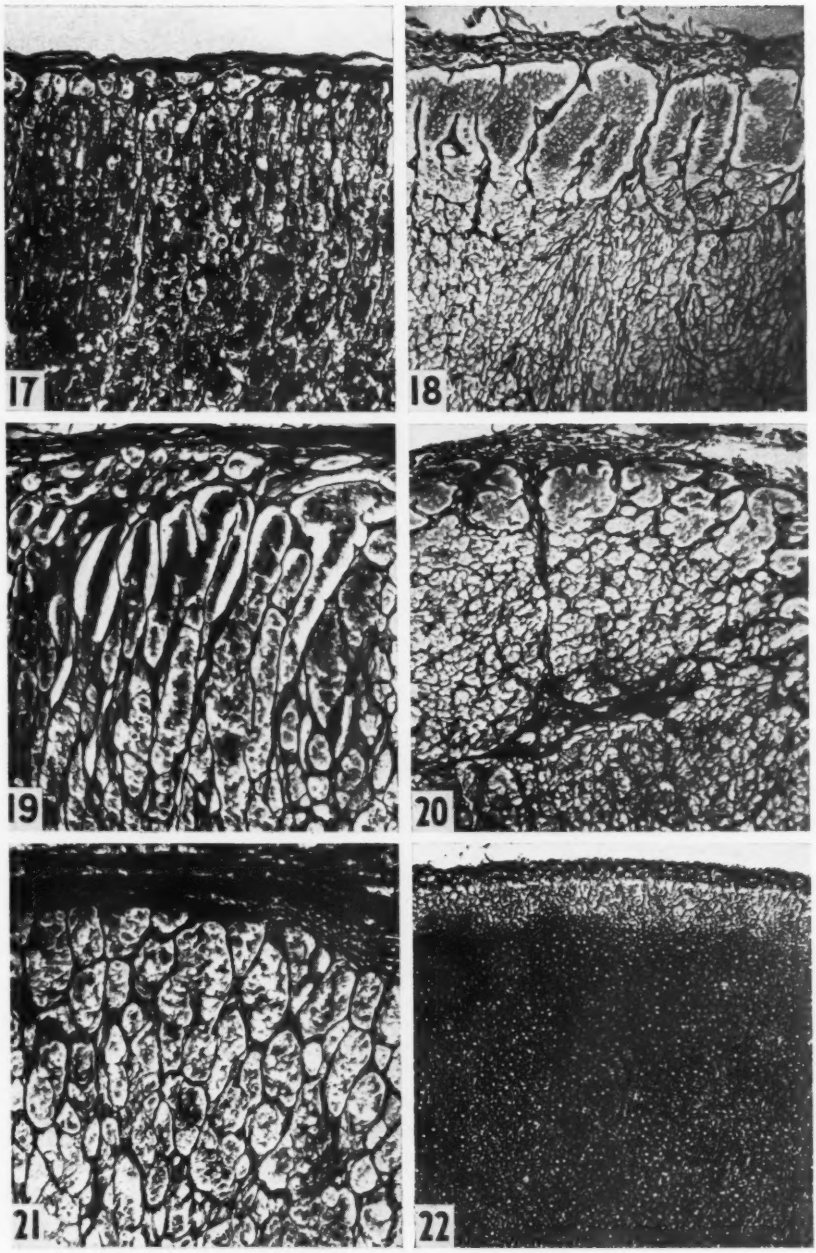
Zona reticularis: In the zona reticularis the fuchsinophil tissue is of very varied abundance. It may be so massive and dense that it seems to form an internal capsule between cortex and medulla. Again it may be scanty. Its essential structure can be studied best in cases in which it is abundant. In slides stained with Masson's trichrome mixture the connective tissue of this zone presents an almost continuous mass interspersed with long, wavy lines (Fig. 25), the sections of corrugated membranes very similar to those found in cirrhotic livers. But a better idea of its structure is obtained with the van Gieson stain. In such slides one sees a finely fibrillar feltwork between the parenchymal cells (Fig. 26). Long, corrugated lines, representing sections of membranes also appear in these masses, apparently as condensation from the finely fibrillar feltwork. It may be suggested that this peculiar, interstitial tissue of the zona reticularis arises through interaction between ground substance and decomposition products of necrotic cells, very much like the process of membranogenesis as suspected to exist in postnecrotic cirrhosis, (Popper and Elias, 1955). In fact one sees frequently a foamy fuchsinophil pattern in the cytoplasm of the necrotic cells just preceding decomposition.

The stroma of the zona reticularis is a finely fibrillar, van Gieson-positive, feltwork in which membranous condensation occurs.

3. *The relations between argyrophil and fuchsinophil tissue*

In the capsule the pattern of collagen fibers appears identical with that of the argyrophil fibers in the light microscope. This phenomenon can be interpreted in three different ways: 1. The argyrophil fibers may be thought to lie between the collagen ones forming the same pattern. 2. The argyrophil fibers may be thought to be coated with a film of collagen. 3. The correct answer seems, however, to be implied in recent electron microscopic studies: Wyckoff (1952) showed that the characteristic periodicity of collagen fibrils is due in part to periodically arranged cavities within the fibers. Schwarz and Pahlke (1953) observed fibers stainable with the van Gieson reaction which can also be impregnated with silver. They found little black beads within the collagen fibers arranged in the form of individual inserts exhibiting the same periodicity as Wyckoff's cavities. These observations suggest that the fibers of the adrenal capsule are collagen fibers which can be silvered internally and periodically.

If the zona glomerulosa is studied at high magnification in slides impregnated with silver a fibrous pattern comes into view. The argyophil fibers of this zone are of varying thickness. The thicker ones are usually straight, indicating a certain stiffness. They do not form networks, but cross each other at various angles. However, very fine fibers seen in the same zone may be curved and appear to be stuck together forming a delicate flat network between the thicker, stiff fibers. They may perhaps branch and form true networks. But light microscope observations are too



FIGS. 17 to 22. Cross sections of adrenal cortices, impregnated with silver (Gomori method): 17. Guinea pig, 18. dog, 19. man, 20. calf, 21. cat, 22. rat.

coarse to recognize the true relationship between these very fine fibers. This structure has been beautifully illustrated by Comolli (1908). The arrangement can be observed at those places where a cross section of the gland is directed tangentially to one of the septa described by Gerlach (1848) and by Arnold (1866) (Fig. 28). In tangential sections of the cortex one sees rows of transverse and oblique sections of the argyophil fibers (dots and commas) (Fig. 27a) while a similar section stained with picrofuchsin shows long lines at the same location (Fig. 27b). The same septa, then, contain criss-crossings and networks of fibers and fuchsinophil membranes. Occasionally, one succeeds to counterstain a silver impregnated slide with van Gieson (Fig. 28). In such cases it becomes obvious that the septa of the zona glomerulosa consist of crisscrossing argyrophil fibers, in the spaces between which fuchsinophil membranes are suspended, just as a soap solution may form a film in the meshes of a wire net which had been dipped into it.

From a morphological and mechanical point of view, these fiber-reinforced membranes can be compared with twine netting imbedded in plastic sheets frequently used for windows of chicken houses. Also the membranes of the zona fasciculata are reinforced by argyrophil fibers. But these fibers are not confined to the membranous sheath. Instead they project out of them to form the reticular baskets that penetrate between the individual parenchymal cells; since those intercellular fibers are purely reticular and not membranous.

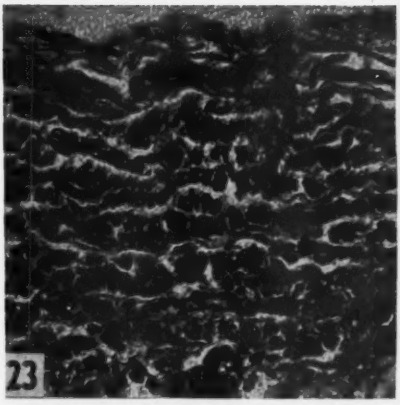
To recapitulate: the capsule contains collagen fibers (threads) running parallel to the surface of the organ. These fibers are, probably, internally argyrophil.

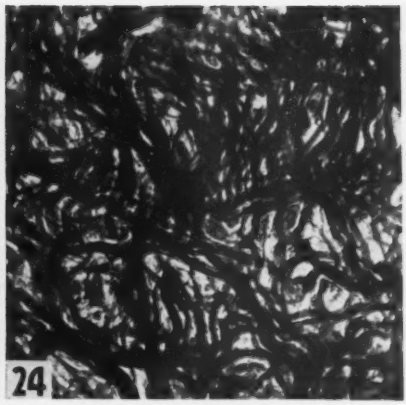
The stroma of the zona glomerulosa consists of flat septa. These are formed by fuchsinophil membranes reinforced by crisscrossing, argyrophil fibers. They enclose rather large compartments containing parenchymal masses. The argyrophil fibers of the glomerulosa do *not* enter into the parenchymal, intercellular spaces.

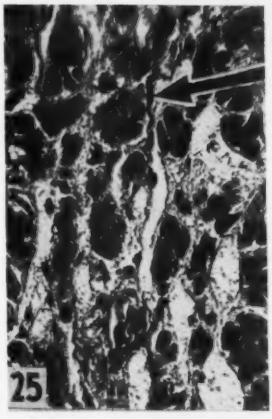
The stroma of the zona fasciculata forms reinforced, membranous tubes which ensheath the parenchymal cell cords and plates. Argyrophil networks form basket-like envelopes around the individual cells of this zone.

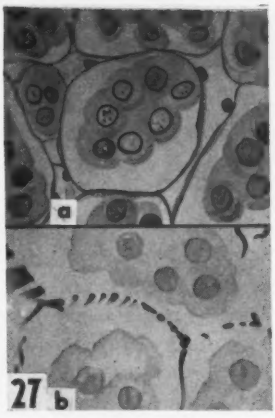
Vasculature

The blood vessels of the adrenal cortex have been described very adequately by Flint (1900) for the dog, Lever (1952) for the rat and by Gersh and Grollman (1941) for the rat and mouse. For the present study, the vasculature of the human adrenal cortex was studied by means of India ink injections and by reconstruction from serial sections. The findings of the previous workers could be verified. As the stroma, so does also the vasculature maintain a rather constant pattern in all mammals studied. Gersh and Grollman have created an excellent stereogram of the blood









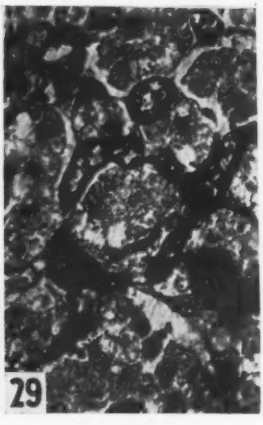


Fig. 23. Stroma of human adrenal cortex: vertical section of capsule, van Gieson.

Fig. 24. Capsule, tangential section, van Gieson.

Fig. 25. Zona reticularis, Masson's trichrome.

Fig. 26. Zona reticularis, drawing from van Gieson preparation.

Fig. 27. Tangential sections of zona fasciculata: a. stained with van Gieson; b. impregnated with silver (Gomori) drawings.

Fig. 28. Vertical sections of zona glomerulosa impregnated with silver, counterstained with van Gieson, showing reinforced membranes: a. drawing; b. photomicrogram.

Fig. 29. Tangential section of zona fasciculata showing the cross sections of India ink injected capillaries.

vessels of the cortex. Yet, for the sake of completeness a stereographic sketch of the vasculature is shown in Figure 2. There is a subcapsular, arterial plexus. From it, capillaries run downward into the zona glomerulosa forming baskets with oblong meshes. Neighboring baskets have common walls. The capillaries widen as they reach the zona fasciculata. They become so wide that neighboring capillaries frequently touch each other (Figs. 1 and 29). Upon reaching the zona reticularis, the capillaries became thinner again and usually bend all in approximately the same direction entering the medulla at acute angles (Fig. 16). In all the zones neighboring vertical capillaries anastomose with one another. There are also arterial loops dipping into the cortex and returning into the subcapsular plexus. Also medullary arteries exist which run directly through the cortex to enter the medullary capillary network without giving rise to cortical capillaries. All these things have been observed and described by Bennett and Kilham in the cat (1940), Gersh and Grollman in the rat and mouse (1941), Harrison in the rabbit, rat and cat (1951), Lever in the rat (1952) and others. We can confirm them for the human species.

In the zona glomerulosa and fasciculata the capillary basketworks are located in the reinforced membranous septa as shown in Figures 1, 3, 27a and 39.

In the zona fasciculata the membrano-fibrous sheets surrounding the enlarged capillaries are more delicate than the septa of the glomerulosa. In the zona reticularis the capillaries are thinner and are embedded in the feltwork of this zone (Fig. 16).

Parenchyma

While the stroma and vasculature of most mammals show a remarkable uniformity, the parenchyma of adrenal cortices of various species, of various individuals within a species and of various regions of the same gland is probably the most variable histological entity known.

The outermost portions of the adrenal cortex are the object of a controversy which has divided observers into two camps. One side presented by Gottschau (1883), Bachmann (1954), Zwemer (1936), Zwemer, Wooton and Norkus (1938), Gruenwald (1942), Gruenwald and Konikov (1944) and Elias (1948) advanced the idea of cortical cell replacement from nondifferentiated, mesenchymal cells in the capsule. We can call this viewpoint the "capsular blastema" theory. Opposed to it are the various observations by Hoerr (1931), Bennett (1940) and Tonutti (1953) which point to different methods of cell replacement. Prominent among them is the theory of intercortical mitotic cell multiplication (Hoerr, 1931). Fortunately, we are in the position to end the controversy. For both sides are right. The controversy was based on the habit of generalizing which has produced so many unnecessary disagreements among biologists. The adherents of the blastema theory, by a strange coincidence, had studied the

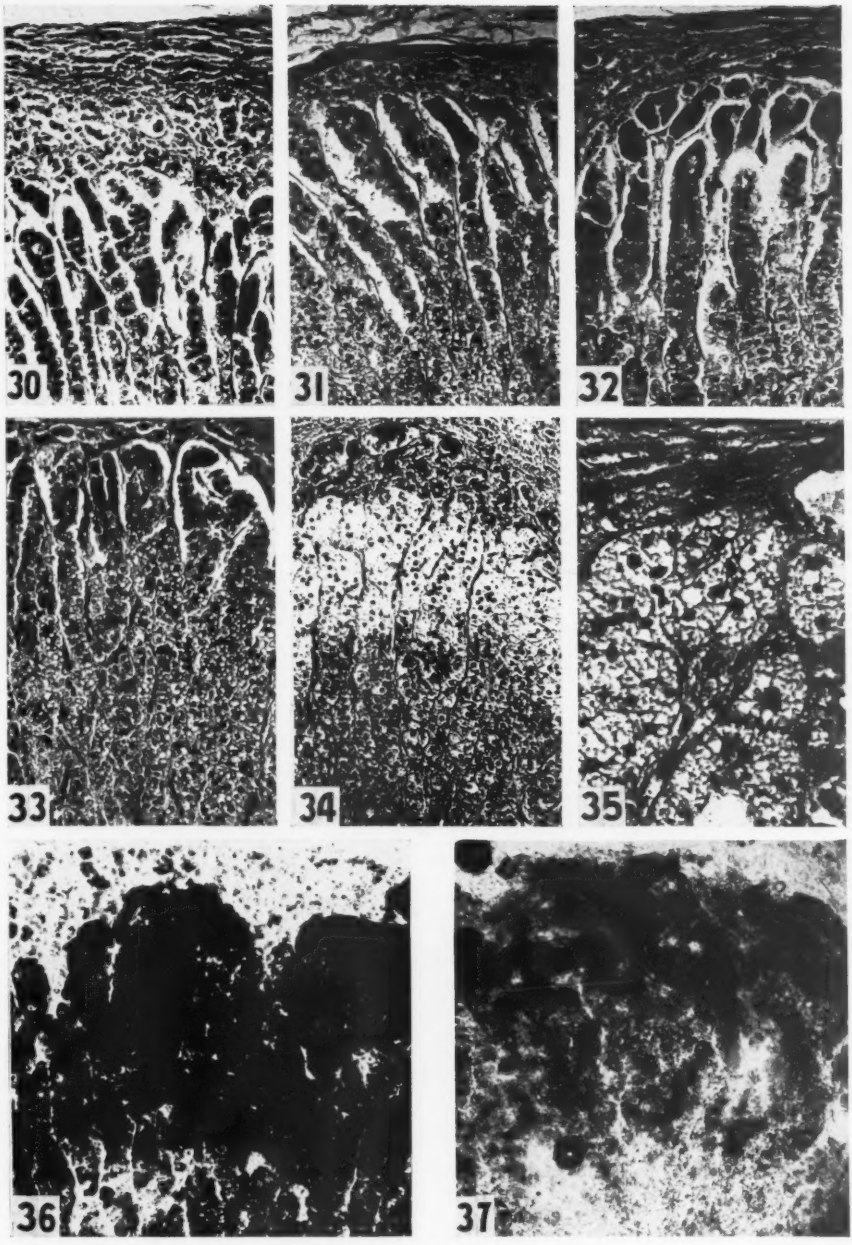


FIG. 30. Typus levis of zona glomerulosa.

FIG. 31. Typus levis.

FIG. 32. Typus levis. For explanation of direction of cutting see text figure 4, right side.

FIG. 33. Typus nodosus.

FIG. 34. Typus lipoidus (Masson's, low power).

FIG. 35. Typus lipoidus, showing lumina in syncytium.

FIG. 36. Typus lipoidus (sudan IV).

FIG. 37. Typus lipoidus (Schultz reaction).

adrenal cortices of animals in which cell replacement seems to be accomplished in a capsular blastema, while the other group worked with animals in which this method of cell replacement plays at the most a very subordinate role. We are in a position to state this because the junior author has just completed a study of the adrenal cortex of the rat, in which there is little if any evidence at all of a capsular blastema. We are in reality not dealing with diverging opinions on the same subject, but with observations on different species which have strikingly different properties.

Man belongs to those mammals in which the intracapsular blastema is conspicuous.

Parenchymal cell groups occur in abundance in the capsule (as defined and identified by its fibrous connective tissue, by the absence of membranes and by its position superficial to the large "cavities" of the glomerulosa of the 19th century authors). The more superficial cells in these groups are small and dark staining. Those located deeper are of larger size and resemble the cells of the glomerulosa more closely, the closer one approaches the zona glomerulosa.

In all of the 63 glands which were observed, many of them in serial sections, we have not seen one single mitotic figure in the cortex; however, it must be admitted that absence of mitoses in postmortem material cannot be considered as very conclusive.

Parenchymal Zonation

It cannot be emphasized too strongly or too often that parenchymal cells located in the fibrous capsule do not belong to the zona glomerulosa if we are to maintain the classical definition of zones by Arnold. And there is no reason not to maintain it, although even Bachmann, the rediscoverer of the intracapsular blastema has made the mistake of labeling cell group in the capsule "zona glomerulosa" and of labeling the zona glomerulosa "upper part of zona fasciculata," in Figure 89 of his recent monograph (1954).

In the following description cell groups located entirely in the fibrous capsule are designated as blastema. However, the blastema will only be considered as far as it is connected with the zona glomerulosa. It will not be described for itself.

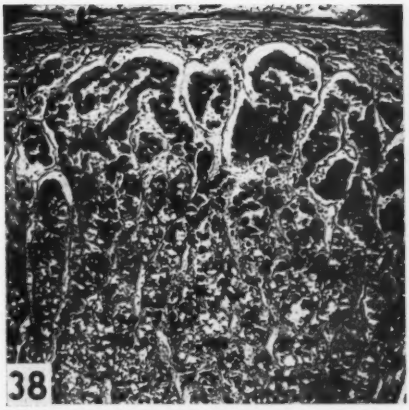
The parenchyma of the zona glomerulosa consists of those cell groups which are located in the large cavities of this zone as defined by the reinforced membranous septa which enclose them.

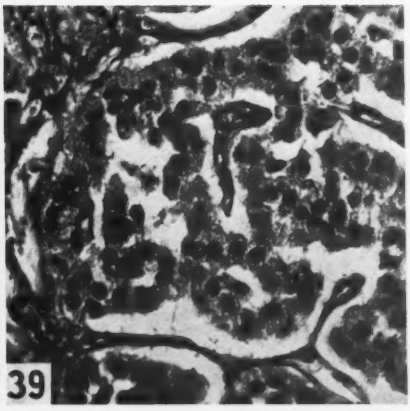
The parenchyma of the zona fasciculata consists of cells individually surrounded by argyrophil fiber baskets.

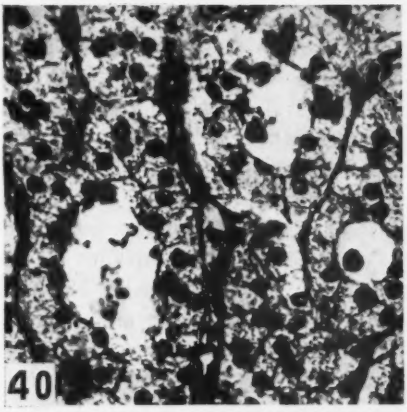
The parenchyma of the zona reticularis is found deep to these cells.

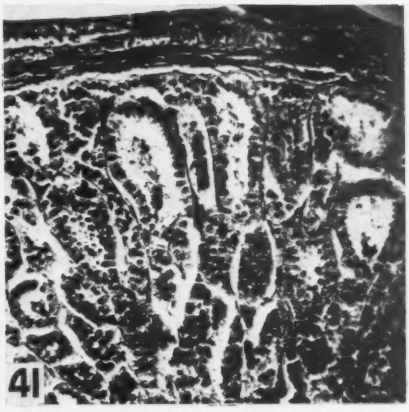
Zona Glomerulosa

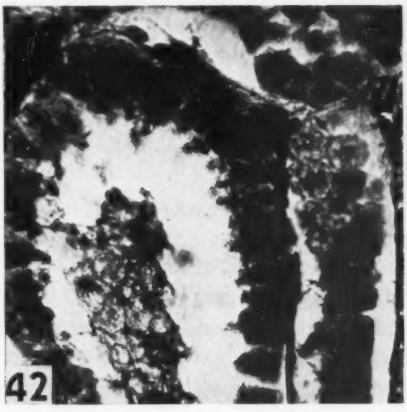
This is the most variable zone of the adrenal cortex, not only from species to mammalian species, but from individual to individual and from place











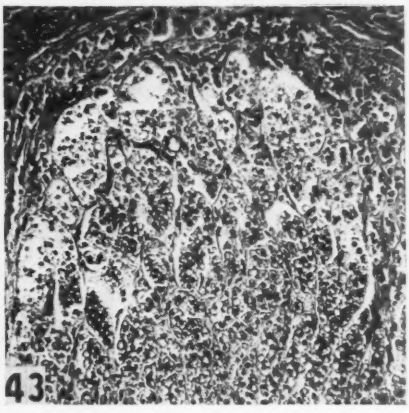


FIG. 38. Typus aperto-follicularis bovinus (low power).

FIG. 39. Typus aperto-follicularis bovinus (high power).

FIG. 40. Typus clauso-follicularis, common form (notice nuclear polarity).

FIG. 41. Typus clauso-follicularis, unusual form (low power).

FIG. 42. Typus clauso-follicularis, unusual form (notice nuclear polarity and apparently ciliated cells).

FIG. 43. Typus aperto-follicularis atrophicus.

to place within one gland. The description of the zona glomerulosa, therefore, constitutes the major topic of this paper. Many different structural types have been found. The types will be designated by descriptive adjectives. They are listed in the order of their approximate frequency.

Typus levis (*smooth type*)

Straight or smooth cords, bands or plates are the most frequently encountered and morphologically the simplest kind of parenchymal cell group of the glomerulosa (Fig. 30). These smooth, oblong portions of parenchyma are often straight and perpendicular to the capsule. Frequently, however, they are directed obliquely to the capsule (Fig. 31). Often they are curved like half parabolae becoming tangential to the capsule at their apices. Neighboring smooth cords, bands or plates are always parallel to each other. If one is lucky enough to cut in the plane of the parabolae, one can see these parenchymal cords in longitudinal section (Fig. 31). More frequently however the section is oblique or perpendicular to the plane of these parabolae. In the latter case the cords will appear in transverse section, that is, almost circular, near the capsule; and they will appear oval further down (Fig. 32). The axial ratio of their sections will increase with their distance from the capsule. In sections perpendicular to the capsule but oblique to the planes of the parabolae, individual cords will appear as horizontally placed ovals near the capsule. For an explanation of these geometrical relations the reader is referred to Figure 4.

The smooth cords and plates are not always as regular as in Figures 30, 31 and 32 but show individual variations, as is seen in the reconstruction Figure 5. They are never isolated from one another, but always connected with each other by parenchymal bridges, so that the entire region forms one parenchymal continuum. The connecting bridges pass through perforations in the reinforced vasculomembranous septa. The cords of *typus levis* consist in most cases of small, isodiametrical cells of low lipoid content.

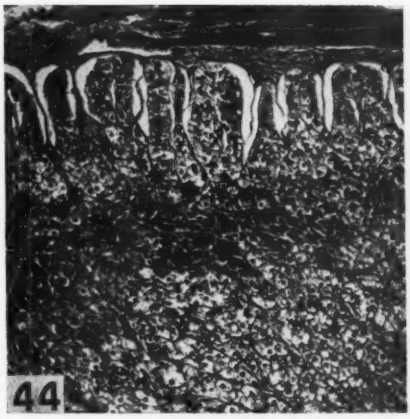
At the capsular side, the smooth cords are always connected with irregularly crooked cords of blastema cells (Fig. 5).

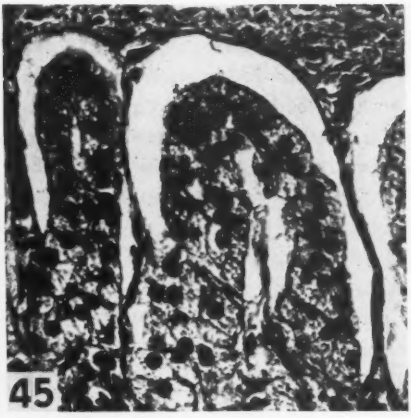
Typus nodosus (*knotty type*)

Irregularly crooked cords, bands and plates (Figs. 6 and 33) with frequent connections occur less frequently than the first type mentioned. They form three dimensional networks. The cords, bands and plates consist of small isodiametrical cells, poor in lipoid. The parts of these knotty networks are usually two cells thick; but at occasional locations they are only one cell thick. The parts of this type are always connected with cord like cell groups of the blastema.

Typus lipoidus (*fatty type*)

Large masses of cells rich in lipoid fill the stromal cavities of the zona glomerulosa and consist of large cells with a foamy cytoplasm filled with





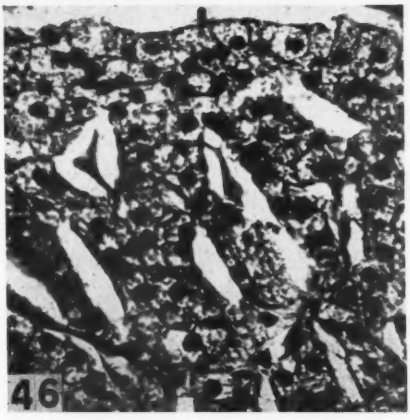


FIG. 44. Typus arcuatus caninus (low power).
FIG. 45. Typus arcuatus caninus (high power).
FIG. 46. Typus muraliosus.

closely packed, small (approximately 2 μ in diameter) lipoid vacuoles. The fatty content of these vacuoles is shown by sudan IV stain in Figure 36. The presence of cholesterol esters and of steroids in these vacuoles is shown by the Schultz reaction in Figure 37. (This reaction gives negative results in the perinephric adipose tissue in the same sections).

In the same region in which we find great accumulations of lipoid in the zona glomerulosa, the zona fasciculata is devoid or almost devoid of lipoid (Fig. 34). This finding was made with great consistency in the majority of the specimens. It is contradictory to the statements of textbooks of histology that say that the greatest lipoid accumulation exists in the zona fasciculata. These statements are based on findings in the cat (Bennett 1940), but not on observations on human adrenal cortices.

One of the most important findings of this study was this realization that

in most areas of human adrenal cortices more lipoid and more cholesterol esters are found in the zona glomerulosa than in the zona fasciculata.

The lipoid laden masses of *typus lipoidus* are frequently of a cellular structure as is clearly evident from visible cell boundaries. Almost equally often, however, these masses appear to have a syncytial character. Cell boundaries, in these cases (Fig. 35) are not recognizable, and one finds large areas devoid of nuclei and at other places accumulations of many nuclei.

Lumina of various sizes occur frequently in parenchymal masses of this type (Fig. 35).

The cell masses of typus lipoidus are always connected with their neighbors by parenchymal bridges. They are also connected in every case with cell groups of the blastema (Fig. 7).

Typus aperto-follicularis bovinus (*calf like type of open sacs*)

This type consists of large, irregularly folded laminae which are one cell thick and are composed of cuboidal cells poor in lipoid with centrally located nuclei. The laminae are one cell thick and resemble a simple, cuboidal epithelium. They form follicles which are always open (Figs. 8 and 9 and Figs. 38 and 39) in such a way that the lumen of each follicle is in direct communication with the perifollicular tissue space. These open follicles resemble those which are regularly encountered in cattle (Elias 1948; Weber, McNutt and Morgan, 1950). In no case has a blastema been observed overlying open follicles. This is the only recorded type above which no blastema occurs. This type is of infrequent occurrence in healthy persons. In one person with a chronic disease used in the preliminary survey it was the predominant type. Neighboring follicles are continuous with each other by means of parenchymal bridges.

Typus clauso-follicularis (*type of closed cavities*)

There exist large cell masses in the zona glomerulosa of very irregular shape which contain lumina of various sizes (Fig. 10 and 40). Such a lumen which may be oblong in shape upon reconstruction, may be shown to be rounded at one end, while at the other end it appears to be wide and collapsed (Fig. 10). The lumina are closed at all sides. No direct communication with the perifollicular space exists. In open follicles the nuclei of the cells are located in their centers, as was shown; since direct communication of the internal with the external space exists, there is no cellular polarity. In closed follicles the lumen is separated from the surrounding tissue spaces. Hence, there is a physiological polarity. We are therefore not astonished to find this polarity expressed in a striking excentricity of the nuclei of the cells which bound the lumen. The nuclei touch the lumen and frequently protrude into it (Fig. 40). Cell masses containing closed follicles are usually rich in lipoid, are always continuous with neighboring

cell groups and are connected with an abundantly developed blastema.

In one case, a large area was encountered which consisted mainly of large, closed follicles (Figs. 41 and 42). Their walls are made of a simple, high cuboidal or low columnar epithelium with marked nuclear polarity, the nuclei being located near the lumina. The borders of the cells adjacent to the lumina show a distinct ectoplasmic condensation and high projections which may be cilia (Fig. 42). Upon reconstruction (Fig. 11), one notices that all these follicles are parts of one continuum. This parenchymal continuum contains plates (Fig. 41, right of center; Fig. 11, left of center) in which the nuclei are located in the cellular centers. In the stereogram (Fig. 11) the interparenchymal spaces are exaggerated for the sake of clarity.

Typus aperto-follicularis atrophicus (*type of degenerating open sacs*)

This structural type consists of open follicles with walls one cell thick. The shrunken cells are applied to the surrounding membranous septa (Figs. 12, 43). A small amount of blastema consisting of small cells exists above these atrophic follicles. Connections between the blastema cells and the atrophic follicles do not exist. This type occurs in the same specimens which contain the bovine type of open follicles.

Typus Arcuatus Caninus (*dog like arched type*)

As this name indicates, there occurs a type of zona glomerulosa which resembles the zona arcuata of the dog. For a comparison with that species the reader is referred to Figure 297 in Sharpey-Schafer's "Essentials of Histology." It consists of large, folded laminae of the structure of a simple columnar epithelium (Figs. 13, 44 and 45). It differs however from a typical columnar epithelium, since its tall, prismatic cells contain spherical (rather than oval) nuclei. The cells are rich in lipoid. In sections, a single folded lamina which extends over a large area of cortex appears as a series of arches.

Typus muraliosus (*type of connected walls*)

This type consists of continuous masses of cells tunneled by a labyrinth of lacunae which contain vascularized, reinforced, membranous tubes. This is an exceptional structure in as much as the vascularized connective tissue occurs in the form of cylindrical sleeves surrounded by parenchyma. The labyrinth of lacunae is in communication with the periparenchymal space. The parenchymal portions separating neighboring lacunae are usually one cell thick (Figs. 14, 46). The name *typus muraliosus* is chosen because of its similarity in basic structure to liver parenchyma which has been called a *muralium* (wall work, system of connected walls). The muralia consist of cuboidal cells rich in lipoid.

* * *

Intermediate forms between all the above enumerated basic types occur. The transitions are fluent. Neighboring cell groups of the glomerulosa, even if pertaining to different types, are always continuous with each other by parenchymal bridges. Therefore, the zona glomerulosa forms one uninterrupted continuum.

Any two types named above can occur side by side. Up to six types in one microscopic field at very low power (scanning lens) have been counted.

Various cell types of the zona glomerulosa. There is a great variety also in the structure of the cells composing the zona glomerulosa. Most of these types have been briefly described with the structural types of which they are characteristic. The various types of cells which occur in normal, human zonae glomerulosae are:

1. Small, isodiametrical cells poor in lipoid occur in typus levis, nodosus and aperto-follicularis bovinus.

2. Columnar cells have been found in *typus arcuatus caninus, muraliosus* and in rare instances in *typus aperto-follicularis* (which in the presence of columnar cells cannot be called *bovinus*). The columnar cells are rich in lipoid, but distinguished from columnar cells in other organs by their spherical nuclei and by absence of polarity.

3. Large, isodiametrical cells very rich in lipoid occur in *typus lipoidus.*

4. Syncytia very rich in lipoid occur also in *typus lipoidus,* frequently together with large, isodiametrical cells.

5. Coarsely vacuolated cells not yet mentioned above contain a small number of large vacuoles, two to six on a section of the cell. These vacuoles leave the centrally located nucleus between them. The cytoplasm of these cells is dark staining. They were found in only two specimens and especially in *typus levis* and *typus nodosus.* In both cases, these coarsely vacuolated cells occurred also in the zona fasciculata.

Nuclei. All the nuclei in the zona glomerulosa are approximately spherical. A nucleolus cannot be demonstrated (an important criterion for the distinction of adrenocortical carcinoma from hepatocarcinoma).

When stained with Masson's trichrome stain, nuclei which stain red can be distinguished from nuclei staining blue. No transitions occur. It is the same phenomenon as reported for liver cells by Sheinin and Davenport (1931). Variously staining nuclei occur side by side. No count of the percentagewise distribution was made.

Distribution of the Types. To establish accurately the quantitative distribution of the various types seems to be impossible. One reason is that transitions between types are frequent. Also it is not possible, when studying one section or even 50 serial sections, to know which types occur in other parts of the same gland. However, a very rough analysis of each section was made as accurately as the material and the judgment of the observer permitted. The results are entered in table 1.

Zona Fasciculata

The cell groups of the zona glomerulosa, regardless of type, go over into the cords of the zona fasciculata, splitting at their deep side. The cells of the zona fasciculata are, indeed, arranged in cords or cylinders, as the textbooks correctly state. This can be verified; in tangential sections of the cortex one sees the round cross sections of these cords or cylinders. One to five cells may be seen on a cross section of a cord (Figs. 1, 27a and 29).

Occasionally (in a total of three glands of our material, and there only

in limited areas) the cells of the fasciculata may be arranged in two cell thick plates, 10–20 cells wide (Fig. 15). These plates are not connected with their neighbors.

The cords (or plates) of the zona fasciculata are surrounded and separated from each other by vascular sheaths. These consist of the large capillaries of this zone which are surrounded by delicate, fiber-reinforced membranes. The arrangement is shown in Figures 1, 27a and 29.

Zona Reticularis

The cords of this zone are thin, their cells are dark staining and the nuclei frequently pyknotic. Upon reconstruction, it can be shown that the cords may branch and that neighboring cords are connected with each other. In the lower part of this zone, isolated and necrotic cells can be found. The large spaces between the cords are frequently filled with a finely fibrillar connective tissue in which membranous condensations occur. The amount of connective tissue is extremely variable from specimen to specimen. In specimens with little fibrillar tissue, there are large tissue spaces in their stead. The capillaries in this zone are relatively thin. They form a network, but do not frequently touch the cell cords nor each other. There exists a controversy on the role of this zone. Some observers believe that it is a zone of degeneration as Mulon (1903) has postulated, while others deny it; most prominent among them is Tonutti (1953). One should not forget that Tonutti's object of observation is the guinea pig, and it may well be that he is right as far as that animal is concerned.

Figure 16 shows a stereogram of the zona reticularis in a case of abundant fibrillar tissue. This figure illustrates the thiness of the cords and their large mutual distances. This drawing is based on sections stained with Masson's trichrome stain. In this stain, the parenchyma is sharply distinguished from the connective tissue. However, most observers use the hematoxylin and eosin stain. And in that case, stroma and parenchyma look alike; therefore the zona reticularis appears very much like a liver. One sees a continuum of red closely applied to the capillaries. Since connective tissue of this very delicate, fibrillar type stains uniformly red, it can not be distinguished in hematoxylin-eosin preparations from the parenchymal cytoplasm. It is probably for this reason that some authors deny the existence of cellular necrosis in this region; for the very small, shrunken size of the degenerating cells cannot be noticed in hematoxylin-eosin stained slides.

SUMMARY

The structure of adrenal cortices of 49 normal human beings, killed accidentally, judicially or by suicide was studied applying three-dimensional criteria.

TABLE 1

Case No.	Source	Cause of death	Adrenal	Typus levis	Typus nodosus	Typus lipoidus	Typus aperto-follicularis bovinus	Typus clauso-follicularis	Typus aperto-follicularis atrophicus	Typus arcuatus caninus	Typus muralis osus
7-2-54	C.C.H.	A	I	+++	+	++	+				
			II	++	++	+	+				
22-2-54	C.C.H.	A	I	+++	+	+					
			II	+++	+	+			++		
24-2-54	C.C.H.	A	I	++	+	++	+	+	+		
			II	+		+++			+		
32-2-54	C.C.H.	A	I			+++	+			+	
						+++	+			+	
52-2-54	C.C.H.	A	I			+++	+			+	
			II			+++	+		+	+	
63-2-54	C.C.H.	A	I	++		+++	++	+			
			II	+++	+	+	++				
65-2-54	C.C.H.	A	I	++	++	+	+				
			II	++	+	++					
73-1-54	C.C.H.	A	I	+++	+	++		+		+	+
			II	+++		+				+	
118-1-54	C.C.H.	A	I	++	+	+++	+				
			II	+++	+	+++	+	+			
123-1-54	C.C.H.	A	I	+++	+	+			+		
			II	+++	+	+	+				+
126-1-54	C.C.H.	A	I			+	++		+		
			II			+	++		++		+
147-1-54	C.C.H.		I	++		+	++		+		
			II	+++	+	+					
153-1-54	C.C.H.	A	I	++	+		+	+			+
			II	++	++		+	+			+
162-1-54	C.C.H.	A	I	++	++	+	+	+			
			II	++	+	+	+	+			
520383	AFIP	AE	I	+		+++	++	+			
532720	AFIP	AE	I	+	+++		+			+	
562657	AFIP	AE	I	+	+++	+	+				
566393	AFIP	AE	I	++	++	++					
566418	AFIP	AE	I	++	+++	+	+	+			
567957	AFIP	AE	I	++	+	+	+				
570347	AFIP	AE	I	+	+++	+	+	+			
574267	AFIP	AE	I	++	++	+	+				
579779	AFIP	AE	I	++	++	+	+	+			
582781	AFIP	AE	I	++	+	+	++		+		

Since left and right adrenals could not always be distinguished the numbers I & II have been used to designate contralaterals.

Code:

+ Present
++ Abundant
+++ Predominant

SGSW = Suicide-gun shot wound
SH = Suicide-hanging
JH = Judicial hanging

A Accident
AE = Accidental Electrocution
JE = Judicial Electrocution

TABLE 1—*continued*

Case No.	Source	Cause of death	Adrenal	Typus levis	Typus nodosus	Typus lipoidus	Typus aperto-follicu-laris bovinus	Typus clauso-follicu-laris	Typus aperto-follicu-laris atrophi-cus	Typus arcu-atus caninus	Typus murali-osus
597736	AFIP	AE	I	++	++	+	+	+			
599269	AFIP	AE	I	++	++	+	+				
599639	AFIP	AE	I	++	++	++	+				+
632364	AFIP	AE	I	++	++	++	+	+			
639084	AFIP	AE	I	++	++	+	+				+
524398	AFIP	AE	II	+	++		++				
236674	AFIP	JE	I	+++	+	+		+			
525624	AFIP	SH	I	+++		++				+	
512298	AFIP	SH	I	++			+		++	++	
509043	AFIP	SH	I	+++		++	+	+	+	++	
502547	AFIP	SH	I	+		+++				++	
490310	AFIP	SH	I	+	+++	+					
482946	AFIP	SH	I	++	++	+	+	+			+
115299	AFIP	SH	I	+++	+	+	+		+		
122547	AFIP	SH	I	++	+++	+	+	+	+		+
224794	AFIP	SH	I	++	+++	+		+			+
106860	AFIP	JH	I	++	++	+	+				
544807	AFIP	SGSW	I	++	++	+	+	+			
197640	AFIP	SGSW	I	++	++	+	+				
169122	AFIP	SGSW	I	++	++	++	+	+			
152367	AFIP	SGSW	I	++	++	+	+				
163045	AFIP	SGSW	I	++	++	+	+				
523041	AFIP	AE	I	+	+++	+	+				
524398	AFIP	AE	I	+	+++	+	+				+
530295	AFIP	AE	I	++	+++	+	+	+			

Stroma. The connective tissue of the capsule is fibrous. In the zona glomerulosa it consists of strong, fuchsinophilic membranes reinforced by argyrophil fibers. The connective tissue of the zona fasciculata is composed of delicate, reinforced membranes and free, intercellular, argyrophil fibers. The stroma of the zona reticularis is a very delicate, fibrillar feltwork concontaining a few membranous condensations.

Vasculature. A subcapsular, arterial plexus gives rise to cortical, vascular basketworks.

Parenchyma. The zona glomerulosa shows a great variety of structural types. These have been named as follows: typus levis, typus nodosus, typus lipoidus, typus aperto-follicularis bovinus, typus clauso-follicularis, typus aperto-follicularis atrophicus, typus arcuatus caninus and typus muraliosus. These types may occur side by side in the same gland. In most cases and in most places the greatest lipoid and steroid accumulations are found

in the zona glomerulosa. The zonae fasciculata and reticularis consist mainly of connected cords. The entire cortex is one continuum of cells.

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INDISPENSABILITY OF THE ADRENALS FOR THE NEPHROSCLEROTIC ACTION OF HYPOPHYSEAL PREPARATIONS[1]

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WHETHER the somatotrophic hormone (STH) exerts any adrenal-mediated actions is still much debated. Many STH effects are certainly not dependent upon the integrity of the adrenals. For instance, somatotrophin can stimulate skeletal growth, proliferation of lymphatic tissues and inflammatory granuloma formation, even in adrenalectomized rats, provided the animals receive adequate maintenance therapy in the form of salt-supplements or corticoids. Yet, it has never been possible, either with purified STH preparations or with crude lyophilized anterior-pituitary (LAP) tissue, to produce nephrosclerosis in adrenalectomized rats. The inhibition of this particular STH effect by adrenalectomy is always complete, even in rats optimally "conditioned" for nephrosclerosis (by unilateral nephrectomy and NaCl supplements), and maintained in good health with adequate amounts of adrenocortical extract (Dontigny, Béland, Hall, and Selye, 1946; Hall, Dontigny, Béland, and Selye, 1946; Selye, 1946), cortisone (Selye, 1951a, 1951b), or cortisone plus desoxycorticosterone (Selye, 1952).

It is especially noteworthy in this connection that desoxycorticosterone does produce nephrosclerosis, both in intact and in adrenalectomized rats; yet even this action of the mineralocorticoid can be aggravated by STH only if the adrenals are intact (Selye, 1951c) and not after adrenalectomy (Selye, 1953).

All these observations suggested that, unlike most of its classic actions, the nephrosclerotic effect of STH depends upon the functional integrity of adrenal tissue; it is abolished after adrenalectomy, even if both gluco- and mineralocorticoids are supplied by injection.

The STH preparations we used for this work were virtually devoid of any contamination with ACTH (as judged by their inability to stimulate the adrenals of hypophysectomized rats); yet they cause marked adrenal enlargement in intact animals (Selye, 1951c), presumably by stimulating endogenous ACTH production. It was reasonable to suspect, therefore,

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that the STH-nephrosclerosis—being abolished by adrenalectomy—might be mediated through a secondary discharge of ACTH from the pituitary. However, ACTH itself proved to possess remarkably little nephrosclerotic action (even in the presence of the adrenals) and certainly much less than STH (Selye, 1950). Still, the possibility existed that STH might produce nephrosclerosis through a dual action: by influencing the kidney directly, as well as through the pituitary-adrenal axis (stimulation of ACTH and glucorticoid secretion). Indeed, since not all the effects of the available ACTH preparations are mediated through the adrenals (Jacot and Selye, 1952), we even had to consider the possibility of an extra-adrenal synergism between STH and ACTH. Finally an extra-adrenal synergism between STH and ACTH, if it occurred, might still presuppose the simultaneous presence in the body of corticoids as "conditioning factors."

In order to provide most favorable experimental conditions for the study of any possible synergisms between STH and ACTH, the two hypophyseal hormones were administered (singly and in combination) both to intact and to adrenalectomized rats, which were simultaneously treated with adequate maintenance doses of 17-hydroxycorticosterone and desoxycorticosterone.

From these experiments we hoped to learn two things:

(1) Whether the nephrosclerotic action of STH can be increased by ACTH in the presence of the adrenals. This was of interest, since we had already demonstrated that the nephrosclerotic action of mineralocorticoids can be increased by simultaneous treatment with glucocorticoids (Selye, 1950).

(2) If conjoint treatment with STH and ACTH should prove to be particularly nephrotoxic, our experiments could be expected to show whether the intense effect of this pituitary-hormone combination could still be abolished by adrenalectomy, even in animals supplied with adequate (but not in themselves markedly nephrotoxic) quantities of both mineralo- and glucocorticoid hormones.

MATERIALS AND TECHNIQUES

Fifty female Sprague-Dawley rats were subdivided into five groups, each consisting of ten rats, whose initial mean body-weight was 90 gm. (range: 84–97 gm.). On the first day of the experiment in all five groups the left kidney was removed through a subcostal incision, under ether anesthesia. In Group V (cf. Table 1) bilateral adrenalectomies were performed simultaneously.

Throughout the experiments all the animals were fed on "Purina Fox Chow" and received exclusively 1% NaCl to drink.

Both cortisol acetate (COLA) ("Cortril," Pfizer Laboratories) and desoxycorticosterone acetate (DOCA) ("Cortate," Schering Corporation) were administered subcutaneously (in the form of microcrystals in aqueous suspension) at the daily dose-level of 100 μg. in 0.2 ml. of water. Although only the adrenalectomized animals of Group V actually needed this corticoid treatment for life maintenance, it was administered, for uniformity's sake, to the rats of all groups.

ACTH ("Duracton," Nordic Biochemicals Ltd.) was given at the dose of 5 I.U. in 0.25 ml. of water (with carboxycellulose as a retarding agent) twice daily subcutaneously, throughout the experiment, to Groups II, IV and V. STH ("Somatotropin," The Armour Laboratories) was administered to Groups III, IV and V at the dose-level of 3 mg. in 0.2 ml. of water subcutaneously, once daily, during the first ten days, the dose being doubled during the rest of the experiment.

The fluid-intake of the animals was measured daily, as an indication of the polyuria which is a characteristic clinical sign of hormonally-produced nephrosclerosis. All the animals were killed with chloroform on the 33rd day; their organs were fixed in Susa mixture, weighed and histologically examined on hematoxylin-eosin and on PAS-stained paraffin sections.

RESULTS

The general design of the whole experiment is summarized in Table 1, together with the principal findings.

The *body-weight* was considerably increased in all three groups which received STH. This increase was markedly diminished by simultaneous treatment with ACTH (Group IV). It is clear that this anti-anabolic effect of ACTH is adrenal-mediated, since—unlike the anabolic action of STH—it failed to occur after adrenalectomy, despite adequate corticoid-substitution therapy (cf. Group V).

The most striking increase in relative *renal-weight* (mg. of kidney tissue per 100 gm. of body-weight) was observed after conjoint administration of ACTH and STH in the presence of adrenal tissue (Group IV).

It is noteworthy that, at the doses given, combined treatment with COLA and DOCA in itself sufficed to produce traces of *nephrosclerosis* (appraised in a scale of 0 to 3 plus) in the control rats (Group I), which received no pituitary-hormone treatment. This shows that the basic corticoid treatment—which was supposed to act as substitution therapy in the adrenalectomized animals of Group V—was not only sufficient for maintenance, but actually somewhat nephrotoxic in itself. Since these renal lesions were very slight we cannot conclude with certainty, from their absence in Group V, that adrenalectomy significantly inhibited this effect of the corticoids, under our experimental conditions. It is quite clear, however, that the nephrotoxic action of ACTH and STH was summated when both hormones were administered concurrently in the presence of the adrenals (Group IV), and that even the resulting intense nephrosclerotic action of the combined pituitary-hormone treatment was completely prevented by the removal of the adrenals (Fig. 1).

The changes in *cardiac weight* are more difficult to interpret. Expressed as a percentage of the body-weight, the cardiac weight—like the renal weight—was highest in Group IV, but not significantly higher than in any of the other groups, except the adrenalectomized rats (Group V). It is interesting, however, that adrenalectomy induced such a remarkable diminution of the relative cardiac weight in the rats receiving both ACTH and STH.

TABLE 1. INDISPENSABILITY OF THE ADRENALS FOR THE NEPHROSCLEROTIC ACTION OF HYPOPHYSEAL PREPARATIONS

Group	Treatment*	Final body-weight (gm.)	Kidney			Heart			Adrenals (mg.)	Thymus (mg.)	Spleen (mg.)	Fluid-intake (ml.)‡
			Weight		Nephrosclerosis	Weight		Myocarditis				
			(mg.)	(mg./%)†		(mg.)	(mg./%)†					
I	—	173	1513 ± 64.0	875 ± 24.3	0.4	730 ± 31.3	423 ± 15.1	0	53.2 ± 2.6	276 ± 29.0	473 ± 19.0	100 ± 4.0
II	ACTH	148	1453 ± 80.1	982 ± 33.0	1.0	612 ± 22.9	416 ± 12.4	0.5	104.5 ± 2.9	60 ± 14.3	300 ± 25.8	112 ± 4.0
III	STH	247	2615 ± 128.0	1060 ± 44.1	1.6	1004 ± 38.3	407 ± 12.6	0.2	84.6 ± 5.4	491 ± 78.6	859 ± 34.9	151 ± 9.5
IV	ACTH + STH	194	2353 ± 123.1	1230 ± 74.4	2.5	833 ± 27.9	437 ± 21.5	2.0	121.2 ± 4.3	58 ± 13.6	421 ± 38.8	204 ± 10.6
V	ACTH + STH + Adr-X	255	2094 ± 172.4	820 ± 56.4	0	971 ± 65.3	381 ± 21.2	0	—	715 ± 63.1	814 ± 58.9	88 ± 6.4

* In this column only those treatments are mentioned which differed in the various groups. As explained in the text, the rats of all five groups were "conditioned" for nephrosclerosis (by unilateral nephrectomy and 1% NaCl) and received 100 μg. of COLA and 100 μg. of DOCA daily.

† Relative organ-weight, expressed per 100 gm. of body-weight.

‡ Mean fluid-intake during the last ten days of observation.

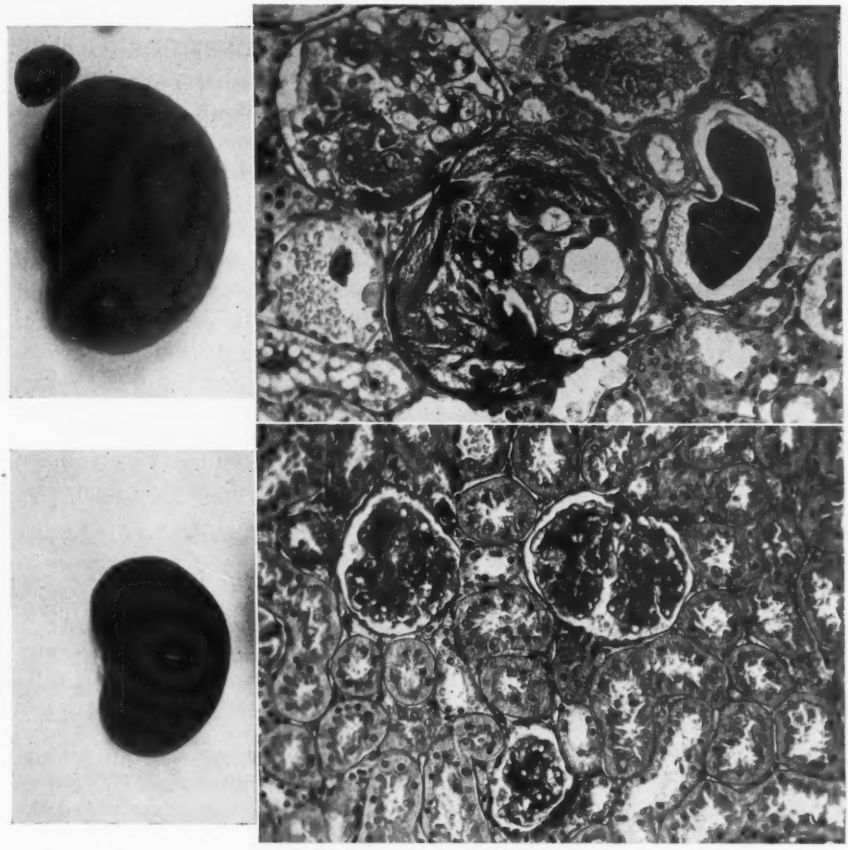

FIG. I. Effect of adrenalectomy upon nephrosclerotic actions of hypophyseal preparations.—*Top:* On the left, macroscopic view of the greatly enlarged, spotty kidney and the hypertrophic adrenal of a rat treated simultaneously with ACTH and STH. On the right, histologic appearance of same kidney. Partly hyalinized and inflamed hypertrophic glomeruli and tubule containing hyalin cast (Group IV). *Bottom:* Macroscopic and microscopic appearance of kidney in adrenalectomized rat receiving the same hormonal treatment. Note complete absence of pathologic changes (Group V).

Signs of *myocarditis* were slight and inconstant among the animals receiving either ACTH or STH alone, but very pronounced in those given both pituitary hormones. In this respect again, the effect of combined treatment with ACTH and STH was totally abolished by adrenalectomy.

The *adrenals* were also largest in Group IV and significantly ($P<0.01$) heavier than in Group II, which received ACTH only. However, in comparison with the controls (Group I), STH in itself likewise produced a marked and highly significant ($P<0.01$) adrenal enlargement.

In agreement with expectations, the weight of the *thymus* was increased by STH (Group III), and this effect was counteracted by the thymolytic action of ACTH (Group IV). In the adrenalectomized animals, however, STH was even more thymotrophic than in intact rats, despite simultaneous treatment with ACTH (Group V).

The weight of the *spleen* roughly paralleled that of the thymus.

Fluid-intake, which served as an indicator of *diuresis*, was barely significantly augmented by ACTH and markedly raised by STH, but neither of these hormones was as effective in this respect as combined treatment with both hypophyseal principles. Adrenalectomy totally abolished the intense polyuria caused by conjoint treatment with ACTH and STH (Group V).

DISCUSSION

The most important outcome of these experiments was that adrenalectomy prevents the intense nephrosclerosis normally produced by combined treatment with STH and ACTH. This inhibition was always complete

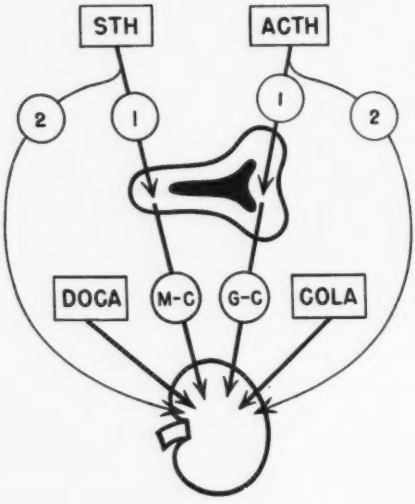

FIG. 2. Diagram illustrating the hormonal condition created in our experimental animals.—The exogenously administered hormones were: ACTH, STH, COLA and DOCA. It is assumed that the adrenal cortex also produces endogenous glucocorticoids (G-C) which act like cortisol and mineralocorticoids (M-C) which act like desoxycorticosterone. We must establish for both hypophyseal hormones (given singly or conjointly) whether they act on the kidney exclusively through the adrenals (route "1") or whether, in the presence of exogenous DOCA and COLA, they could also act directly.

even though the rats were optimally "conditioned" for nephrosclerosis (unilateral nephrectomy, sodium chloride) and received adequate substitution therapy with both 17-hydroxycorticosterone and desoxycorticosterone. To evaluate this finding in the light of earlier observations, it may help to depict schematically (Fig. 2), the rather complex hormonal situation which we had to create in our animals.

Earlier work had shown that the nephrosclerotic action of steroids depends primarily on their mineralocorticoid potency. Pure glucocorticoids do not have this effect themselves, but they augment the nephrosclerotic action of simultaneously injected mineralocorticoids (Selye, 1950).

It had also been shown that STH imitates DOCA in its ability to produce nephrosclerosis but, unlike the mineralocorticoids, growth hormone exerts its nephrotoxic action only in the presence of the adrenals. In hypophysectomized rats, whose adrenal cortex is atrophic, STH is almost devoid of nephrosclerotic potency, unless ACTH is simultaneously given. Yet, hypophysectomy does not protect the kidney quite as completely against STH nephrosclerosis as adrenalectomy does (Ventura and Selye, 1955–56). From all this it was tentatively concluded that STH may act upon the kidney through the intermediary of the adrenals; indeed, the hormone is apparently dependent for this effect upon a fairly well-developed adrenal cortex, as judged by its virtual inactivity after hypophysectomy.

We therefore felt that optimal conditions for the production of nephrosclerosis would be achieved by treating suitably "conditioned" rats simultaneously with both types of pituitary hormones, as well as with both types of corticoids. This experimental arrangement had the further advantage that, even after adrenalectomy, the animals remained supplied with both mineralo- and glucocorticoids. We argued that if STH has any not adrenal-mediated, nephrosclerotic effect (route "2"), this should manifest itself even if it were dependent upon the simultaneous presence in the body of ACTH and either or both kinds of corticoids.

The doses of corticoids given here were sufficiently high to produce slight nephrosclerosis by themselves and ACTH caused pronounced adrenal enlargement. Under these conditions, simultaneous treatment with STH induced extraordinarily severe nephrosclerosis, polyuria and myocarditis in intact animals and yet, no trace of such effects could be detected after adrenalectomy. This finding is compatible with the view that both the growth hormone and ACTH act upon the kidney exclusively through the adrenals (route "1"), presumably by stimulating the secretion of some corticoid principle(s).

In any event the nephrosclerotic effect of STH is not merely a consequence of its anabolic action, as adrenalectomy did not inhibit the stimulation of growth by STH although it prevented the production of renal damage.

The possibility still remains, however, that STH acts directly upon the kidney, but only in the presence of some corticoid which cannot be replaced by COLA and DOCA. Corticosterone is not likely to be this steroid, since it does not produce nephrosclerosis even when given at relatively high dose-levels to optimally "conditioned" rats (Selye and Bois, 1954).

Another finding worthy of comment was that even ACTH alone aggravated the nephrosclerosis of intact rats receiving conjoint treatment with COLA and DOCA. Some years ago—on the basis of similar experiments on rats (using nephrosclerosis as an indicator of mineralocorticoid activity)—we had concluded that ACTH is rather ineffective in stimulating the mineralocorticoid production of the adrenals (Selye, 1950). This has since re-

peatedly been confirmed—both in animals and in man—by identifying the corticoids in the blood and urine after ACTH treatment (for literature cf. Selye and Heuser, 1955–56). Presumably, the nephrosclerosis observed in Group II of the present experimental series was due to the glucocorticotrophic effect of ACTH, since we had shown that glucocorticoids aggravate nephrosclerosis in DCA-treated rats.

The marked adrenal enlargement caused by our virtually ACTH-free STH preparation also deserves comment. In these, not hypophysectomized, rats this hypertrophy may have been due to ACTH secretion in response to injected STH; if so, the still greater adrenal enlargement in the animals receiving both STH and ACTH would actually be due to summation of exogenous and endogenous ACTH actions. It should be remembered, however, that injections of COLA, DOCA and ACTH have all been shown to diminish endogenous ACTH secretion. The corticotrophic effect of STH—if it were merely relayed through an increased endogenous ACTH production—would therefore have to be so considerable as to overcompensate for the inverse effects of all the three other hormones given in Group IV. In other words, under these experimental conditions, STH is either corticotrophic itself or it so intensely stimulates the production of ACTH (or of some other endogenous corticotrophin) that it overcomes the inhibitory action of the injected ACTH and corticoids.

All these observations make it highly probable that STH does exert certain adrenal-mediated actions upon the kidney. Yet it is still possible that these actions of the growth hormone are not direct corticotrophic effects, but actions relayed through some other organ, for instance, the pituitary.

SUMMARY

Rats, "conditioned" by unilateral nephrectomy and sodium-chloride supplements, received cortisol and desoxycorticosterone, in doses just sufficient to produce traces of nephrosclerosis.

Under these conditions of maximal sensitization for nephrotoxic actions, ACTH alone or STH alone sufficed to cause manifest nephrosclerotic lesions. When simultaneously given, the two pituitary hormones synergized each other in this respect.

Even the intense nephrosclerosis (accompanied by myocarditis), induced by combined treatment with STH and ACTH in these highly sensitized rats, was completely prevented by adrenalectomy, despite the substitution therapy with both 17-hydroxycorticosterone and desoxycorticosterone.

It is concluded that the nephrotoxic effect of STH is either relayed through the adrenal (due to some special, not ACTH-like, corticotrophic effect), or dependent upon the simultaneous presence in the body of some adrenal hormone which cannot be replaced by adequate maintenance doses of cortisol and desoxycorticosterone. Our experiments do not exclude the

possibility that this adrenal-mediated STH effect is an indirect corticotrophic action relayed through some other organ, for instance, the hypophysis.

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EFFECT OF HORMONES ON THE *IN VITRO* INCORPORATION OF GLYCINE-1-C^{14} INTO RETICULOCYTE PROTEINS[1]

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IT IS known that many steroid hormones affect both protein metabolism (Rutgers University Protein Symposium, 1953) and the formed elements of the blood (Gordon, 1954). Thus, the administration of ACTH results in an increase in urinary amino acids (Ronzoni *et al.*, 1953) and adrenalectomy of the eviscerated rat causes a decrease of plasma amino acids (Ingle *et al.*, 1950). Barton and Rusch (1951) have shown that administration of adrenocortical extract inhibited the *in vivo* incorporation of glycine-2-C^{14} into several tissue proteins. Similarly, it has been found by Sinex (1954) that prior treatment of intact rats with ACTH will cause a decrease in the *in vitro* incorporation of alanine-1-C^{14} into liver slices. However, Lee and Williams (1952) found that adrenalectomy reduces the *in vivo* incorporation of cystine-S^{35} and that this reduction can be restored by adrenocortical extract, cortisone, and desoxycorticosterone. White and Dougherty (1945) have presented evidence that adrenocortical hormones enhance erythropoiesis and Piliero *et al.* (1950) have shown a decreased percentage of developing erythrocytes in marrow following adrenalectomy. However, little is known of the mechanisms involved. A study involving the reticulocyte could encompass both the maturation process of the erythrocyte and protein metabolism. In the present paper the *in vitro* effect of various hormones on the incorporation of glycine-1-C^{14} into the proteins of reticulocytes has been described.

METHODS

To induce reticulocytosis, mature male New Zealand rabbits were injected subcutaneously daily for two successive days with 1.0 ml. of a 2.5% neutralized solution of phenylhydrazine hydrochloride. Ten milliliters of blood were withdrawn daily from an ear vein beginning on the day of the first injection; on the 6th to 9th day blood was taken for incubation purposes. Before use, the blood cells were separated from the plasma, washed twice with isotonic NaCl, and brought back to the initial whole blood volume with isotonic NaCl.

All incubations contained 1.0 ml. of the washed blood cell suspension, 0.10 ml. of 0.15 *M* glucose made up in 0.11 *M* phosphate buffer at pH 7.50, 0.10 ml. of glycine-C^{14} containing 3.0 μM glycine and about 370,000 cts./min., 0.10 ml. of an antibiotic solution containing 1.5 mg. each of streptomycin and penicillin G per ml., and hormone or saline up to 1.4 ml. or 1.5 ml. total volume.

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The isolation and washing of the protein for counting purposes has been previously described (Koritz and Chantrenne, 1954). The washed protein was plated from a 15% acetone suspension onto aluminum planchets and dried in an oven at 80° C. The protein was counted in a windowless counter. All planchets were corrected for self absorption and the specific activities expressed as counts per minute at infinite thickness.

Suspensions of the hormones, with the exception of somatotrophic hormone (STH) which was dissolved in saline, were prepared by grinding the material in 0.1% bovine serum albumin (Armours, Fraction V) in a hand operated, all glass Potter-Elvehjem homogenizer. The suspension formed is sufficiently stable to permit accurate pipetting. Control incubations contained equivalent amounts of the serum albumin. Test incubations indicated that neither the serum albumin nor the antibiotics affect the rate of incorporation. When testing substances other than DOC, flasks including two concentrations of DOC were simultaneously incubated to check any variation in the blood cell preparation. The inhibition caused by 0.1 mg. of DOC in six runs varied from 15% to 29% with a mean of 21%. Similarly at 0.5 mg. the range was 87% to 90% with a mean of 89%.

RESULTS

It may be seen from Table 1 that the steroid hormones have either an inhibitory effect or no effect at all. In no case was there any stimulation.

TABLE 1. EFFECTS OF VARIOUS STEROIDS ON THE INCORPORATION OF GLYCINE-1-C^{14} INTO THE PROTEINS OF RABBIT RETICULOCYTES

Substance	mg./1.4 ml.	% Inhibition	Substance	mg./1.4 ml.	% Inhibition
DOC*	0.1 0.5	20 87	Testosterone	0.1 0.5	6 6
DOC acetate	0.6	77	Estradiol	0.02 0.2	0 0
Cortisone	0.1 0.6	6 14	Cholesterol	0.1 0.7	0 0
Cortisol	0.1 0.6	7 8	Pregnenolone	0.1 0.5	0 0
Corticosterone	0.1 0.5	13 53	3β,21-Dihydroxy-Δ^5-pregnen-20-one	0.1 0.5	0 25
11-Dehydrocorticosterone	0.1 0.5	4 24	9α-Fluoro-cortisol	0.1† 0.5†	5 11
11-Desoxycortisol	0.1 0.6	6 9	17α,21-Dihydroxy-$\Delta^{1,4}$-pregnadiene-3,11,-20-trione	0.1† 0.5†	0 18
20β,21-Dihydroxy-Δ^4-pregnene-3,20-dione	0.1 0.5	29 38	ACE‡	0.34† 3.40†	14 95
21-Hydroxyallopregnane-3,20-dione	0.1 0.5	27 66	Progesterone	0.1 0.5	3 13
3β,21-Dihydroxyallopregnan-20-one	0.1 0.5	13 35			

* We are indebted to Drs. K. Pfister and M. Tishler of Merck and Co. and Dr. R. Gaunt of Ciba Pharmaceutical Products, Inc. for generous gifts of steroids.

† Final volume = 1.5 ml.

‡ Adrenocortical extract was kindly supplied by the Upjohn Company. This was an acetone solution of beef adrenal equivalent to 41 lbs. whole adrenals per 41 ml. Before use the acetone was evaporated under N_2. Weights refer to the residual oil which was suspended in saline for use.

Cholesterol and the sex hormones have little or no inhibitory action in this system and only some adrenocortical steroids and their derivatives are active in this respect. Among the adrenal steroids desoxycorticosterone (DOC) is the most potent inhibitor and modification in its structure leads, in general, to a decrease in inhibitory activity. Of the reduced derivatives of DOC, 21-hydroxy-allopregnane-3,20-dione produced the most intense inhibitory effect. Reduction of ring A in DOC to the tetrahydro form (3β,21-dihydroxy-allopregnan-20-one) reduced the inhibitory effect. Reduction of the 20-keto group of DOC to the 20β-hydroxy form resulted in a compound (20β,21-dihydroxy-Δ^4-pregnene-3,20-dione) with high inhibitory action at the 0.1 mg. level (29%) but a marked reduction in activity at the 0.5 mg. level.

The influence of several non-steroid hormones and of various combinations of hormones is seen in Table 2. These substances singly have no effect. On the basis that the inhibitory effects of DOC could be reversed by other hormones or that a synergism existed in this system among hormones, various combinations of hormones were tested. There was no reversal of inhibition nor any synergism among the substances tested. The observed effects of combinations of hormones were an additive function of the individual substances.

TABLE 2. EFFECT OF VARIOUS HORMONES AND THEIR COMBINATIONS ON THE INCORPORATION OF GLYCINE-1-C^{14} INTO THE PROTEINS OF RABBIT RETICULOCYTES

Substance	mg./1.4 ml.	% Inhibition	Substance	mg./1.5 ml.	% Inhibition
Cortisone	0.3	8	DOC	0.1 0.3 0.5	25 76 86
Cortisol	0.3	12			
DOC	0.1	15	STH	0.2	0(+4)*
Corticosterone	0.1	13	STH + DOC	0.2 +0.1 0.2 +0.5	27 85
STH‡	0.2	0	L-Thyroxine†	0.12	0
Insulin‡	0.04	3	L-Thyroxine+STH	0.12 +0.2	0(+10)*
DOC + cortisone	0.1 +0.3	33	L-Thyroxine† + DOC	0.12+0.1 0.12+0.3	16 78
DOC + cortisol	0.3 +0.3	32	Insulin	0.04	0
Corticosterone + cortisol	0.1 +0.3	25	Insulin + DOC	0.04+0.1 0.04+0.5	26 86
STH + insulin	0.2 +0.04	0			
Cortisone + insulin	0.1 +0.04	6			
STH + cortisone + insulin	0.2 +0.1 +0.4	3			

* Added as Na-L-thyroxine.

† A + value indicates stimulation of incorporation above that of the control.

‡ Crystalline Zn-insulin (lot #499667) glucagon free and assaying about 25 units per mg. was obtained through the generosity of O. K. Behrens of the Lilly Research Laboratories. The STH (lot #M-108) was a gift of S. L. Steelman of the Armour Laboratories.

Adrenaline at 0.1 and 0.01 mg. per 1.4 ml. and noradrenaline at 0.20 and 0.02 mg. per 1.4 ml. had no effect on the rate of C^{14} glycine incorporation. l-Thyroxine at 0.1 mg. and 0.5 mg. per 1.4 ml. at 0.5 hr. rather than the usual 2.0 hr. incubation also was without effect. There was no reversal of the ACE inhibition by STH, l-thyroxine or insulin.

DISCUSSION

DOC produced the most intense inhibition with respect to *in vitro* incorporation of glycine-1-C^{14} into reticulocyte proteins. All the modifications of the DOC structure investigated resulted in some loss of inhibitory activity at the 0.5 mg. level. The 20β and the Δ^4 reduced (allo) derivatives were as active as DOC at the 0.1 mg. level. Of special interest is the almost complete loss of inhibitory activity resulting from the insertion of the 17α-hydroxyl group. The extremely low inhibitory activity of cortisone and cortisol may be due in part to this structural modification since corticosterone and 11-dehydrocorticosterone which lack the 17α-hydroxyl group but possess an oxygen substitution at C-11 are significantly more inhibitory. The relatively high inhibitory action of 21-hydroxyallopregnane-3,20-dione was not altogether unexpected in view of the findings of Tullner *et al.* (1954) who found this steroid to be capable of inhibiting estrogen-induced growth of the chick oviduct. The phenomena described in this communication and that described by the latter authors bear further comparison since in both instances 21-hydroxyallopregnane-3,20-dione is a more effective inhibitor than progesterone.

The relatively specific *in vitro* inhibitory effects of DOC on various enzyme systems have been found by others (Hayano and Dorfman, 1951; McNaught *et al.*, 1955). It would appear that DOC *in vitro* has a non-specific inhibitory effect on various enzyme systems. However, DOC *in vivo* has also been found to inhibit body and tail growth of young rats (Maassen, 1952), and Friedberg (1950) has shown a depression *in vivo* of S^{35} methionine incorporation into the proteins of several tissues by the administration of dehydrocorticosterone to adrenalectomized rats. Hauschildt and Grossman (1953) also found DOC to inhibit the incorporation of C^{14} glycine into the proteins of rat and human liver slices. However, testosterone, estradiol and cortisone were also inhibitory in their system. It is of interest, and perhaps of consequence, that cholesterol and the sex hormones had little or no inhibitory action in the blood cell system.

Leslie (1952) and Lu and Winnick (1954) have reported the synergistic action of cortisone, STH and insulin on the growth on embryonic tissue cultures. Selye (1955) has shown a synergism between STH and DCA on somatic and hair growth in adrenalectomized rats. There is also the known antagonism between adrenocortical hormones and insulin. However, in the present study, the effect of various hormones in combination were additive only.

SUMMARY

The action of various hormones and related substances on the in vitro incorporation of glycine-1-C^{14} into the proteins of reticulocytes has been investigated. It was found that the adrenocortical steroids had an inhibitory effect while cholesterol and the sex steroids had little or no effect. *l*-Thyroxine, somatotrophic hormone, and insulin were without effect. The influence of various hormones in combination were additive. Among the adrenocortical hormones desoxycorticosterone was the most potent inhibitor. Modifications of its structure, especially the insertion of an hydroxyl group in the 17 position markedly reduced its inhibitory activity. 21-Hydroxyallopregnane-3,20-dione, previously reported to be effective in inhibiting estrogen-induced growth of the chick oviduct, was found to be an effective inhibitor, second only to desoxycorticosterone.

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THE ROLE OF THE ADRENAL AND THYROID IN PROMOTING BLEEDING IN HYPOPHYSECTOMIZED BABOONS (PAPIO URSINUS)

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BLEEDING from the uterus in Primates is usually attributed to withdrawal of estrogen or progesterone or to simultaneous withdrawal of both these hormones. The role of the pituitary in the bleeding mechanism is said to be secondary via the gonadotrophic hormones which regulate the amount of estrogen and progesterone secreted by the ovary. However, Hartman *et al.* (1930) reported that in the hypophysectomized monkey, estrogen-withdrawal bleeding occurred only after the administration of alkaline extracts of the pituitary. These investigators emphasized that, after treatment with their pituitary extract, bleeding could occur without any observable change in the ovaries or uterus. Accordingly, Hartman *et al.* postulated as the direct cause of bleeding the presence of a hormone independent of follicle-stimulating or luteinizing principles contained in the pituitary.

In the present series of experiments in baboons, it will be shown first, that estrogen-withdrawal bleeding does not occur in hypophysectomized baboons; secondly, both the uterus and sex skin of the hypophysectomized baboon are sensitive to treatment with estrogen; thirdly, the reactions in the glands and stroma of the endometrium, consequent on estrogen injections, can be uncoupled from estrogen-withdrawal bleeding; fourthly, an active thyroid as well as an active adrenal are apparently essential for estrogen-withdrawal bleeding in hypophysectomized baboons. Finally, section of the pituitary stalk not only interferes with estrogen-withdrawal bleeding but also with the pituitary mechanism regulating the secretion of gonadotrophic hormones.

MATERIALS AND METHODS

Six, sexually-mature, young, female baboons between five and six years of age, formed the material for this investigation. Prior to hypophysectomy, the animals were under observation in the colony for six months to two years and, during this time, none was subjected to any experimental procedure. Routine daily measurements of the perineal swelling as well as records of the day of onset and duration of menstruation revealed the presence of normal cycles in all baboons.

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In each of five animals, the hypophysis was removed under direct vision by an orbital approach devised by Dr. E. Epstein. In brief, the following procedure was adopted. A portion of the lateral wall of the supra-orbital bone of the right eye was removed to allow for retraction of the eyeball into a lateral position. The skin was then incised along the medial aspect of the eye, the lacrimal duct divided, and the periosteum of the infra-orbital bone exposed. After reflecting the periosteum, the eyeball was retracted laterally and the medial wall of the orbit exposed. The bone of the infra-medial aspect of the orbital fossa was gently chipped away, proceeding from the front towards the back, thereby allowing for greater movement within the orbit. The sphenoid bone, forming the anterior wall of the fossa, was penetrated and the dura mater overlying the pituitary gland displayed. After incising the dura, the pituitary gland was visible and was removed in two or three pieces with the aid of a specially-designed spoon. The operation was deemed complete when the entire gland could be satisfactorily reconstructed from the fragments removed. The diaphragma sella was carefully preserved and all manipulations within the pituitary fossa occurred below the diaphragma. No suction was applied during the process of removing the gland. Loss of blood was minimal and was kept in check by using adrenalin. After removal of the pituitary, the fossa was flushed with physiological saline, the eyball returned to its normal position, the bone of the lateral wall of the orbit replaced, the lacrimal duct anastomosed and the eyelids stitched together with absorbable catgut.

Post-operative edema of the eye reached maximum intensity within 2 to 3 days and thereafter subsided. As the edema subsided, and the catgut resorbed, the eyelids gradually separated and full vision was restored in the eye at the end of three weeks. The success of this technique is apparent from examination of Fig. 2, representing a photograph taken 4 months after hypophysectomy.

Several criteria were used to establish the completeness of hypophysectomy, namely (1) extreme sensitivity of the animals to insulin, coupled with susceptibility to spontaneous attacks of hypoglycemia, during short periods of food deprivation, (2) a precipitous fall in the protein-bound iodine which was maintained at a low level, (3) return of the sex skin to rest, such as occurs in castrated baboons, and permanent suppression of menstruation, (4) inspection of the ovaries and uterus and histological examination of one ovary and of uterine biopsies of two animals between 6 and 10 weeks after hypophysectomy. Final assessment of the completeness of hypophysectomy was made after death from histological examination of serial sections of the sella turcica and of all the endocrine glands. A section of the adrenal gland of B.340, obtained 15 months after hypophysectomy, shows severe atrophy of the cortex and particularly of the zona fasciculata in contrast to the wide adrenal cortex of a normal baboon of comparable age and weight (Figs. 7 and 8).

In a sixth baboon (B.349), the pituitary stalk was sectioned and a silver plate inserted between the pituitary and the diaphragma sella. This experimental procedure also led to permanent suppression of the menses, but spontaneous hypoglycemia did not occur although the protein-bound iodine level fell to a concentration similar to that described in hypophysectomized baboons (Gillman and Gilbert, 1954, Van Zyl, 1955).

The time of onset of experimentally-induced bleeding in all animals was determined by daily inspection of the vaginal orifice and, where necessary, by daily examination of vaginal smears for a minimum period of three weeks following withdrawal of estradiol benzoate. In all cases, however, bleeding was sufficiently profuse to be detected by external examination.

The animals were maintained on the same diet as all other baboons in the colony (Gillman and Gilbert, 1955) but, in addition, received supplements of eggs, meat and bread overnight to guard against unpredictable attacks of hypoglycemia. Owing to their sensitivity to cold, the baboons were housed in a constant temperature room dur-

ing the night but were allowed to exercise in outdoor cages during the day. The animals remained in good health and body weight was maintained throughout the period of observation ranging from one to three years.

At varying intervals after hypophysectomy, the baboons received courses of treatment with adrenocorticotrophic hormone (ACTH), thyroxin and estradiol benzoate. ACTH ("Acton") was obtained from Frederiksberg Chemical Laboratories. According to assays kindly supplied by Dr. Rerup, the ACTH contained no somatotrophic or thyrotrophic hormones and, as will become apparent from our experiments, no gonadotrophic activity. ACTH was administered daily in two divided doses on a body weight basis namely, 1 unit ACTH per kg. body weight per day. The same concentration of thyroxin was used for all experimental animals, namely, 0.2 mg. injected three times weekly. Estradiol benzoate (0.1 mg.) and progesterone (10 mg.) were given as daily, intramuscular injections.

PROCTOCOLS

The behavior of the sex skin in each of five baboons before and after hypophysectomy is represented graphically in Fig. 1. Turgescence of the sex skin was accompanied in all instances by an increase in body weight and deturgescence by loss of a corresponding amount of weight as previously described (Gillman and Gilbert, 1955).

B.332. This baboon, having a basal weight of 12.5 kg., was hypophysectomized during the phase of perineal deturgescence, approximately 4 days after ovulation. Bleeding *per vaginam* was observed 4 days later and continued for 14 days. The perineal swelling continued to deturgesce and eventually underwent complete resorption.

Fifty-three days after hypophysectomy, a course of treatment with estradiol benzoate was initiated and continued for 43 days. The perineal skin responded to this therapy by turgescence and eventually the swelling attained a size comparable with that recorded during a normal cycle prior to hypophysectomy (Fig. 1, B.332). Upon withdrawal of estrogen, the perineal swelling underwent progressive deturgescence. Repeated examination of the vaginal smear for a period of 4 weeks revealed no sign of bleeding.

During the succeeding months, the baboon received no hormonal treatment. Three-hundred-and-fifty-four days after hypophysectomy treatment with ACTH was instituted and continued for 59 days. Throughout this period of treatment, the sex skin remained in a state of rest, indicating that whatever gonadotrophic hormone might have been present in the ACTH, it was obviously inadequate to promote ovarian stimulation. At the end of this period, a course of treatment with ACTH and thyroxin was given for 23 days. Again, there was no evidence of ovarian stimulation. A 15-day course of estradiol benzoate was then commenced in conjunction with ACTH and thyroxin. The perineal skin commenced to turgesce on the third day. The maximum perineal dimensions, achieved on the 13th day, were noticeably less than those attained on the first occasion of treatment

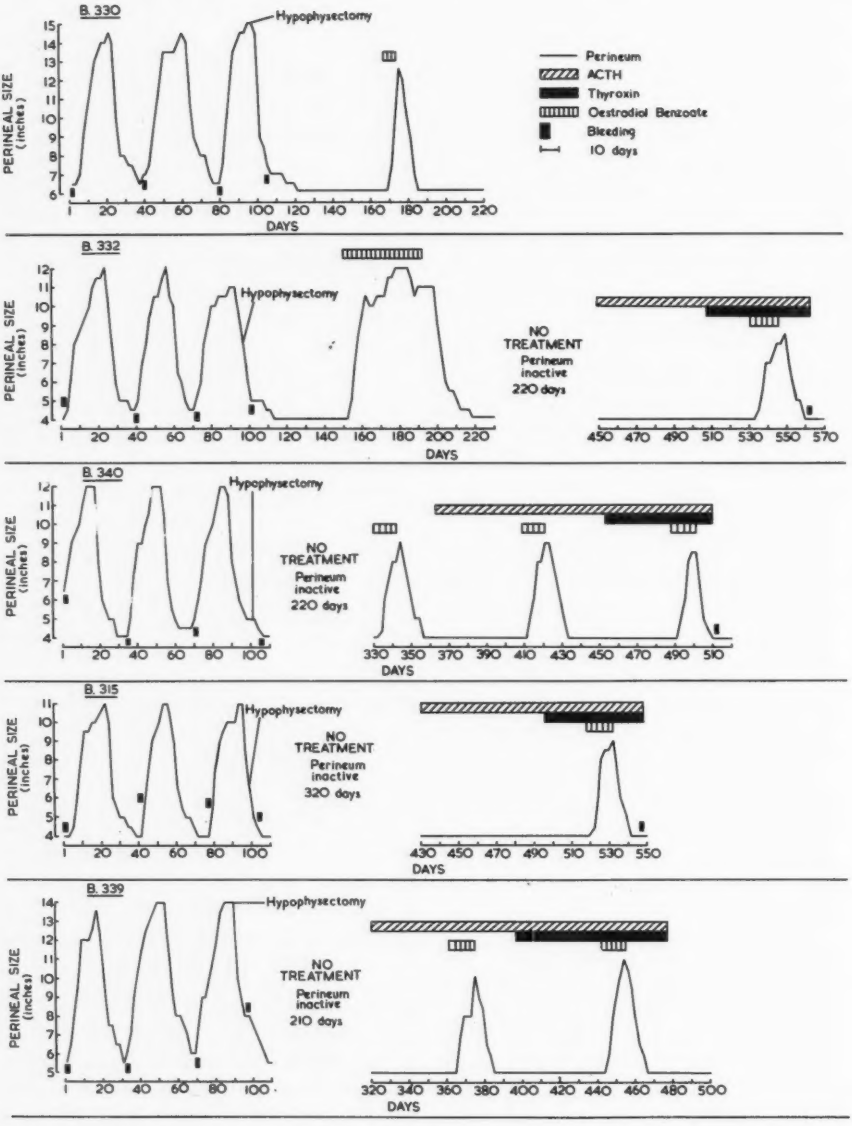

FIG. 1. Response of the sex skin of hypophysectomized baboons (B.332, B.315, B.340, B.330, B.339) to estradiol benzoate administered alone or in conjunction with ACTH or with ACTH and thyroxin.

with estradiol benzoate. After 15 days, estradiol benzoate was withheld but ACTH and thyroxin continued. Bleeding per vaginam was detected on the 14th day after withdrawal of estrogen and continued for 14 days. It is thus apparent that more than one year after hypophysectomy, the administration of estrogen in conjunction with ACTH and thyroxin was able to promote estrogen-withdrawal bleeding.

B.330. This baboon, having a basal weight of 14.0 kg., was hypophysectomized during the phase of maximal perineal turgescence at the time of or on the day before ovulation. The perineal swelling underwent rapid deturgescence and ultimate resorption. Bleeding occurred on the 8th day after the operation and continued for 10 days.

Sixty-nine days after hypophysectomy, the baboon received daily injections of estradiol benzoate for 8 days. The sex skin underwent turgescence until the enlarged sex swelling attained a diameter of 12 inches. Owing to the untoward side-effects induced by estrogen, causing loss of appetite and general malaise, treatment was suspended earlier than planned. No bleeding occurred at any time within the 21 days following withdrawal of estradiol benzoate (Fig. 1, B.330).

B.340. This baboon, having a basal weight of 14.5 kg., was hypophysectomized in the late premenstruum, approximately 15 days after ovulation. Bleeding occurred on the second day after operation at the normally expected time. During the succeeding $7\frac{1}{2}$ months, the animal received no therapy (B.340, Fig. 1).

Two-hundred-and-twenty-five days after hypophysectomy the baboon received a 12-day course of treatment with estradiol benzoate. The perineal swelling reached a maximum diameter of 9 inches, considerably less than that recorded during a normal cycle prior to hypophysectomy. Although examined daily for 21 days following withdrawal of estradiol benzoate, no bleeding was detected.

Treatment with ACTH was then instituted, injections being administered for 46 days. As in the case of B.332 above, ACTH did not evoke any response in the sex skin which could be attributed to the presence of gonadotrophic hormones. After this preliminary treatment with ACTH, daily injections of estradiol benzoate were then commenced in conjunction with ACTH for 13 days. The sex skin responded to the estrogen by turgescence and, as on the previous occasion, the swelling reached a diameter of 9 inches. Estrogen treatment was then withdrawn but ACTH therapy maintained for a further 32 days. During this period, the sex swelling regressed in size and eventually resorbed completely but no bleeding occurred.

It should be mentioned that the animal had now been receiving continuous ACTH therapy for 91 days. At this stage, thyroxin (0.2 mg. 3 times per week) was given in conjunction with ACTH for 35 days. On the 15th

day of combined injections, an unexpected bleeding *per vaginam* was observed and persisted for 3 days. This bleeding was unaccompanied by any change in the sex skin. The treatment with ACTH and thyroxin preceded a 13-day course of injections of estradiol benzoate. ACTH and thyroxin were maintained throughout estrogen treatment and during the period of estrogen-withdrawal. On the 9th day after estrogen withdrawal, bleeding *per vaginam* was recorded and persisted for 6 days.

B.339. This baboon, having a basal weight of 13.1 kg., was hypophysectomized during the phase of perineal turgescence. The perineal swelling underwent rapid deturgescence and, on the 5th day after operation, vaginal

FIG. 2. Face of hypophysectomized baboon showing almost normal appearance of the right eye.

FIG. 3. Section of an ovary removed 73 days after hypophysectomy. A few fluid-filled follicles are still in evidence (×9).

bleeding was detected and continued for 12 days. Five months later the baboon was thyroidectomized.

Two-hundred-and-thirty-six days after hypophysectomy, the baboon received a course of ACTH therapy lasting for 42 days which preceded combined treatment with ACTH and estradiol benzoate for 14 days (Fig. 1, B.339). The sex skin turgesced to a maximum diameter of 9 inches. Withdrawal of estradiol benzoate but maintenance of ACTH was not followed

by bleeding *per vaginam* during the observed period of 22 days. Attention is drawn to the fact that the baboon had now been receiving continuous treatment with ACTH for 78 days. Without interrupting ACTH therapy, thyroxin was administered for 45 days. Estradiol benzoate was then given daily in conjunction with ACTH and thyroxin for 13 days. The perineal swelling turgesced to a diameter of 11 inches. Estrogen was discontinued but ACTH and thyroxin maintained for a further period of 22 days. Vaginal smear examination disclosed no evidence of bleeding during this 22-day period of estrogen-withdrawal.

B.315. Hypophysectomy was performed in B.315, having a basal weight of 17.2 kg., during the phase of perineal deturgescence between 5 and 6 days after ovulation. Bleeding *per vaginam* was observed on the 4th day after the operation (Fig. 1, B.315).

Laparotomy examination on the 73rd day after hypophysectomy revealed an atrophic uterus and very small ovaries. The left ovary was removed and serially sectioned. From Fig. 3 it is apparent that the size of the ovary is greatly reduced in comparison with that of a normal baboon of comparable age (Fig. 4) and the formation of follicles is obviously inhibited. Nevertheless, a number of fluid-filled follicles can be seen, indicating that despite the absence of the pituitary gland, the formation of follicles is not entirely suppressed.

FIG. 4. Section of an ovary of a normal baboon. The baboon was of approximately the same age and weight as the hypophysectomized baboon, the ovary of which is depicted in Fig. 3 (×9).

On the 172nd day after hypophysectomy, the baboon received 10 mg. progesterone daily for 6 days in an attempt to induce uterine bleeding. Withdrawal of treatment was unaccompanied by bleeding within the expected time of 4 to 7 days.

Three-hundred-and-thirty-three days after hypophysectomy, ACTH therapy was commenced and continued for 67 days to be followed by ACTH and thyroxin for 22 days. Estradiol benzoate was then injected daily in conjunction with these two hormones for 14 days only. The sex skin responded to the estrogen by turgescence and attained a diameter slightly less than that recorded in a normal menstrual cycle prior to hypophysectomy. Estrogen was then discontinued but ACTH and thyroxin maintained. On the 14th day after withdrawal of estradiol benzoate, bleeding *per vaginam* was detected.

B.349. The pituitary stalk of B.349 (having a basal weight of 13.8 kg.), was sectioned when the perineal swelling was in a state of maximal turgescence. Six days later, bleeding *per vaginam* was recorded. The sex skin resorbed completely and menstrual cycles were permanently suppressed.

One-hundred-and-seventy-six days after stalk section, the baboon received its first course of treatment, namely, daily injections of estradiol benzoate for 14 days. Although responsive to estrogen, the perineal swelling did not attain the same size as observed during the menstrual cycles prior to stalk section. Withdrawal of estrogen was not attended by bleeding during a 30-day period of observation.

The animal was rested for two months before commencing, simultaneously, injections of thyroxin and estradiol benzoate. The latter hormone was discontinued after 13 days but thyroxin injections maintained for a further 3 weeks. No bleeding *per vaginam* was observed during the 3-week period of estrogen-withdrawal.

Twenty-two months after stalk section, B.349 received a 6-day course (10 mg. daily) of progesterone. On the 5th day after the last injection, red cells were observed in the vaginal smear but there was no evidence of external bleeding.

RESUME OF PROTOCOLS

(a) *Onset of bleeding following hypophysectomy and stalk section*

It is apparent from the data presented above that removal of the pituitary or section of the stalk precipitates uterine bleeding within 2 to 8 days. In three baboons hypophysectomized in the early second part of the cycle, bleeding occurred 4 or 5 days later whereas hypophysectomy just before ovulation in B.330 delayed bleeding until the eighth day. In B.340, the bleeding within 48 hours of hypophysectomy coincided with the time of the normally expected period. Section of the pituitary stalk at ovulation was followed by bleeding within 6 days.

(b) *Failure of estrogen to promote withdrawal bleeding in hypophysectomized baboons*

It has been previously established that the administration of 0.1 mg. estradiol benzoate daily for 10 days to castrated baboons promotes rapid perineal turgescence and stimulation of the uterus, to be followed by deturgescence of the perineum and bleeding 10 to 16 days after withdrawal of the hormone. The same amount of estrogen administered to hypophysectomized baboons also induces perineal turgescence (Gillman and Gilbert, 1953) and promotes growth of the endometrium comparable to that observed in an estrogen-treated castrate (Figs. 5 and 6). Withdrawal of estrogen leads to slow involution of the perineal swelling but, unlike the castrate, is not attended by uterine bleeding either in the hypophysectomized or stalk-section baboons. Microscopic examination of the vaginal smears failed to disclose the presence of any red cells. It is thus apparent that not only the pituitary but also an intact stalk are necessary for uterine bleeding to occur after a period of treatment with estrogen. In this connection, it should be mentioned that estrogen-withdrawal bleeding may fail to occur in long-standing castrated monkeys and baboons. In such instances, withdrawal bleeding can be induced after a course of treatment with progesterone in the monkey (Eckstein, 1950), and in the baboon (Gillman and Gilbert, unpublished data). In the hypophysectomized

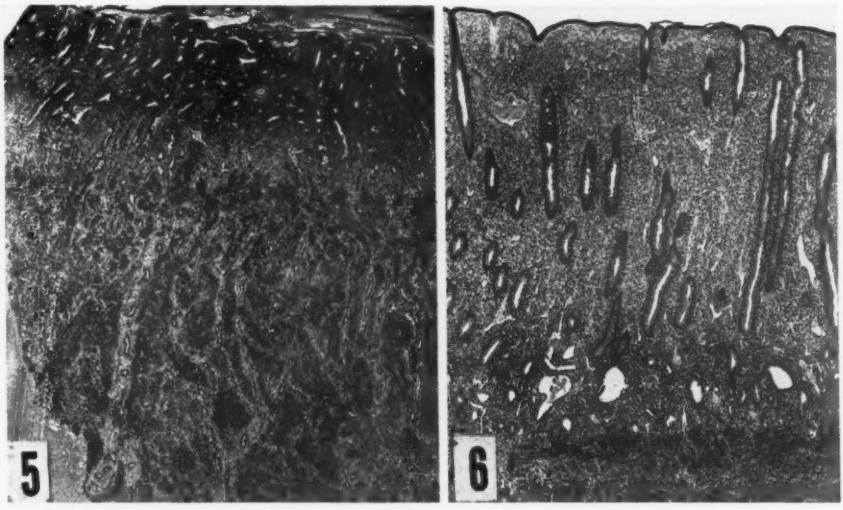

FIG. 5. Section of the uterus showing the atrophic endometrium of a baboon hypophysectomized 73 days previously (×22).

FIG. 6. Section of the uterus of an hypophysectomized baboon following a course of treatment with estradiol benzoate (0.1 mg. daily) for 12 days (×22).

baboon (B.315), however, progesterone, when administered for 6 days, was unsuccessful in evoking bleeding but precipitated scanty hemorrhage in the stalk-sectioned baboon (B.349) on the fifth day after the last injection.

(c) *Effect of ACTH on estrogen-withdrawal bleeding*

The two hypophysectomized baboons (B.339, B.340) receiving ACTH for 6 weeks prior to the commencement of a 13-day course of estradiol benzoate in conjunction with ACTH, failed to show estrogen-withdrawal

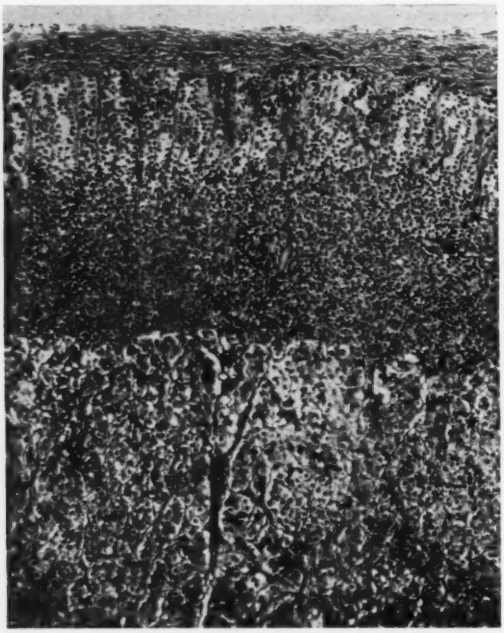

FIG. 7. Section of the adrenal gland obtained 15 months after hypophysectomy. The marked atrophy of the cortex and particularly of the zona fasciculata is striking (×48).

bleeding even in the presence of continued ACTH injections. Stimulation of the adrenal gland alone therefore was unable to induce estrogen-withdrawal bleeding.

(d) *Effect of ACTH and thyroxin on estrogen-withdrawal bleeding*

All the baboons receiving ACTH and thyroxin in conjunction with estrogen, with the exception of the thyroidectomized-hypophysectomized baboon, B.339, showed signs of external vaginal bleeding between 9 and 14 days after withdrawal of estrogen. Both thyroid substitution therapy as well as stimulation of the adrenal cortex are therefore apparently essential for promoting estrogen-withdrawal bleeding in hypophysectomized baboons. No explanation can be offered for the failure to induce bleeding in the hypophysectomized-thyroidectomized baboon (B.339).

(e) *Effect of thyroxin and estrogen in the stalk-sectioned baboon*

Since hypophysectomized baboons deteriorate rapidly when receiving thyroxin treatment alone, it was deemed inadvisable to attempt to examine the effect of pretreatment with thyroxin on estrogen-withdrawal bleeding.

Stalk section in the baboon, as mentioned above, suppresses the menstrual cycle and leads to a sharp decrease in the level of plasma protein bound iodine (PBI) (Gillman and Gilbert, 1954). These observations indicate that continuity between the pituitary and hypothalamus is essential for the maintenance of gonadotrophic and thyrotrophic activity in the baboon as has been demonstrated by Harris (1954) in dogs and cats. Since stalk-section in the baboon does not influence to any extent growth or the capacity of the animal to adapt to sudden changes in temperature and does not necessitate the administration of cortisone or ACTH, it was apparent that the isolated pituitary was able to secrete amounts of ACTH adequate to sustain more than a minimal degree of adrenal function, compatible with survival and certainly greater than that occurring in hypophysectomized baboons. Nevertheless, the amount of ACTH secreted was still inadequate to promote the onset of estrogen-withdrawal bleeding during a course of treatment with thyroxin.

FIG. 8. Section of the adrenal gland of a normal baboon. The wide zona fasciculata occupies the greater part of the field (×48).

COMMENT

At the outset, it should be mentioned that hypophysectomized baboons treated with estrogen suffer a constitutional disturbance manifest as apathy, loss of appetite and excessive discomfort caused by the enlarging sex swelling. Loss of appetite creates a serious problem in view of the rapid appearance of hypoglycemia even after a short period of food deprivation. This constitutional disturbance can be prevented by ACTH. When administered alone to hypophysectomized baboons, ACTH improves the general physical condition and prevents spontaneous attacks of hypoglycemia following food deprivation. Although a loss of body weight occurs during the first 7 to 14 days of treatment with ACTH, the animals thereafter gain weight steadily.

It has been shown above that priming with ACTH and thyroxin is essential for the consistent production of estrogen-withdrawal bleeding in hypophysectomized baboons. It is reasonable to assume therefore that the pituitary gland regulates uterine bleeding not only through its gonadotrophins but also through ACTH and thyrotrophic-stimulating hormone, the latter acting in turn on the adrenal cortex and on the thyroid gland respectively.

Estrogen-withdrawal bleeding can be induced in adult thyroidectomized baboons experiencing prolonged amenorrhea and perineal inactivity (Gillman and Gilbert, 1953). It follows that a physiologically-active thyroid is not a *sine qua non* of estrogen-withdrawal bleeding in baboons possessing an intact pituitary gland. Since estrogen-withdrawal bleeding can be induced in amenorrheic thyroidectomized baboons, it was reasonable to expect that withdrawal-bleeding should appear in ACTH-treated, hypophysectomized baboons, after a course of estrogen. Our experiments have shown that this expectation was not realized. In view of this failure, the possibility was then entertained that the preparation of ACTH used in our experiments contained a factor which promoted bleeding only in the presence of thyroxin. If this were the case, then estrogen-withdrawal bleeding should have occurred in the hypophysectomized-thyroidectomized baboon, B.339, no less than in the hypophysectomized baboons, B.332, B.315, and B.340. However, since B.339 failed to bleed when treated with ACTH and thyroxin prior to a course of estrogen treatment, it can be assumed that the ACTH used in these experiments did not contain a special bleeding factor. It is apparent that in hypophysectomized baboons, both the secretions of the adrenal cortex and of the thyroid gland are necessary for estrogen-withdrawal bleeding. It is equally evident that the mechanism of estrogen-withdrawal bleeding is more complicated than suspected hitherto.

Since it was shown above that both the secretions of the adrenal cortex and of the thyroid gland are essential for estrogen-withdrawal bleeding in hypophysectomized baboons, one further observation deserves consideration, namely the occurrence of bleeding in all baboons within 8 days after

removal of the hypophysis. It is true that extirpation of the hypophysis promptly leads to a precipitous drop in the PBI, but such a drop does not necessarily mean that all the processes modulated by the thyroid hormone are suddenly arrested or depressed. Indeed, we have demonstrated that removal of the thyroid gland from normal, adult female baboons deranges the menstrual cycle at the earliest between 20 and 30 days after the operation (Gillman and Gilbert, 1953). *Per contra*, normal menstrual cycles and the accompanying fluctuations in the sex skin can be re-established in thyroidectomized, amenorrheic baboons only within 2 to 3 weeks after the beginning of substitution therapy with thyroxin. These observations indicate that, even in the face of the rapid decrease in the PBI, the various reactions necessary for bleeding and dependent on the thyroid hormone persist for at least 2 to 3 weeks and, in the case of the hypophysectomized baboon, for at least 8 days after operation.

Comparable evidence relating to continuing effects of adrenocortical hormones after removal of the pituitary gland or of the adrenal glands is also available for baboons. Although the adrenal cortex involutes rapidly after hypophysectomy, cortical activity decreases relatively slowly and only reaches its minimal level between 6 and 24 weeks after hypophysectomy, as judged by the resting blood sugar (unpublished data). Furthermore, bilateral adrenalectomy was followed by uterine bleeding within 7 to 14 days in 4 of 5 baboons investigated to date, indicating that even when the adrenals are removed, the reactions dependent on the circulating adrenocortical hormones and necessary for uterine bleeding continue for at least 7 to 14 days thereafter (Gilbert and Gillman, 1955).

On the basis of the foregoing observations, it would seem that the post-hypophysectomy bleeding reported in the baboon can be explained by the continuing effects of the thyroid hormone in the face of a low PBI and by the fact that adrenocortical activity is still adequate to promote bleeding. However, in the course of time, the marked depression in the function of the thyroid gland and of the adrenal cortex eventually exclude the possibility of inducing estrogen-withdrawal bleeding in hypophysectomized baboons. Our experiments demonstrate that refractoriness to estrogen-withdrawal bleeding is already established by the 50th day after hypophysectomy. But, even if this refractoriness is allowed to persist for 9 months or a year, estrogen-withdrawal bleeding can still be induced by priming with ACTH and thyroxin.

Finally, since the endometrium of the hypophysectomized baboon responds to stimulation with estrogen but fails to bleed when estrogen is withheld, it would appear that the mechanism controlling growth of the endometrium is not necessarily coupled to that regulating bleeding.

SUMMARY

Withdrawal bleeding could not be induced in a series of 5 hypophysectomized baboons or in a stalk-section baboon following treatment with

estrogen alone or estrogen in conjunction with ACTH, even though the endometrium and the sex skin responded to estrogen in a manner similar to that occurring in castrated baboons.

When ACTH was administered jointly with thyroxin prior to a course of treatment with estradiol benzoate, estrogen-withdrawal bleeding was promoted in each of the 3 experimental animals; withdrawal bleeding failed to occur in a similarly treated hypophysectomized-thyroidectomized baboon.

Thyroxin administered to a stalk-sectioned baboon in which adrenal activity was greater than that in hypophysectomized baboons did not induce estrogen-withdrawal bleeding.

It was concluded that while the adrenal and the thyroid undoubtedly participate in some way in regulating uterine bleeding in hypophysectomized baboons, the exact mechanism underlying this regulation still remains to be elucidated.

Acknowledgement

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AN IMPROVED BIOASSAY METHOD FOR THYROTROPIC HORMONE USING THYROID UPTAKE OF RADIOPHOSPHORUS[1,2]

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A RAPID, accurate, and sensitive method is needed for the bioassay of hypophyseal thyrotropic hormone (TSH). Borell, in 1945, demonstrated an increase in total phosphorus content of the thyroid gland following stimulation with TSH. This observation led Borell and Holmgren in 1949 to the use of radioactive phosphorus (P^{32}) uptake of the thyroid of the guinea pig as an indicator of TSH activity. Later, Crooke and Matthews (1953), and Lamberg (1953), presented bioassay methods utilizing P^{32} uptake of the one day old chick. This report describes modifications in the radiophosphorus uptake methods which increase the accuracy and sensitivity of the assay and decrease the work required. Abstracts of this work have been reported previously (Greenspan, *et al.*, 1954; Kriss and Greenspan, 1954).

I. DESCRIPTION OF PRESENT METHOD OF ASSAY

Our standard method for the bioassay of TSH utilizes the uptake of P^{32} by the thyroid of the day-old chick 6 hours after the intracardiac injection of TSH. The details of the method are as follows: one-day-old White Leghorn cockerels[3] are kept in a warm room but are neither fed nor watered prior to the assay. They are picked from the box at random and are numbered serially with a small piece of tape attached to the leg. Standard TSH[4] or unknown solution, dissolved in 0.1 cc. of isotonic saline, is injected directly into the heart and 25 microcuries of P^{32} in 0.5 cc. saline[5] is administered intraperitoneally immediately thereafter. Usually 5 chicks are used for each test dose of standard or unknown solution. Successive groups of 5 animals are placed in separate boxes lined with paper toweling. Six hours later, the animals are killed by decapitation under ether narcosis and the thyroid lobes are removed. The chick thyroid is composed of two separate reddish ovoid structures which lie caudal to the thymus and adjacent to the internal carotid artery on either side of the neck. We usually split the sternum, open the chest widely, and remove the internal carotid artery and thyroid-thymus tissue *en bloc*. The

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[2] Presented in part at the meeting of the Endocrine Society, San Francisco, June, 1954.

[3] Obtained from Poehlman Hatcheries, Petaluma, California.

[4] TSH preparation #R491075 kindly supplied by Armour Laboratories.

[5] P^{32} obtained from Abbott Laboratories.

thyroid lobe is carefully dissected free of thymus tissue under a dissecting microscope. The two thyroid lobes are placed on small copper discs, dried in air, and beta radioactivity is determined by an end-window Geiger Counter. The response is expressed as the square root of T, where T = the time in seconds to count 1024 counts per chick thyroid. Three dose levels of unknown are compared with three dose levels of standard TSH with a 3-fold step increase between each dose. During most of the year our standard TSH doses are 0.25, 0.75, and 2.25 milliunits (1 milliunit equals 0.001 U. S. P. units of TSH). During the months of March, April, and May of 1954 and 1955 we noted that the sensitivity of the chicks diminished slightly, and dose levels of 0.5, 1.5, and 4.5 milliunits of TSH were used. A sample assay curve is shown in Figure 1-A.

II. ANALYSIS OF THE METHOD

1. *Selection of an Index of TSH Activity*

Initially, it seemed desirable to express chick thyroid P^{32} uptake as a ratio between cts./sec./mg. of thyroid and cts./sec./mg. of blood. In these studies, TSH was administered at 4 or 5 dose levels, using 8–10 chicks at each dose level. At the end of the experiment (4–6 hours) the animals were anesthetized with ether, a sample of whole blood was obtained by cutting off the leg and allowing 6–8 drops of blood to fall into a weighed metal cup. The animals were then decapitated, the thyroids carefully dissected out and placed on weighed copper discs. The tissues were allowed to dry for 24 hours and the cups and discs reweighed on an analytical balance accurate to 0.1 mg. Dry tissue weight was obtained by difference. Beta radioactivity was determined in both the dry thyroid and dry blood specimens.

The following measurements were thus obtained: weight and radioactivity of thyroid, weight and radioactivity of blood sample. In any experiment the data could be analyzed in terms of any one of several responses; among these are: (1) Counts per second per thyroid; (2) Counts per second per mg. of thyroid; (3) A ratio of counts per second per milligram of thyroid to counts per second per milligram of blood. Of these possible measures, the first is the simplest; the other two would tend to remove variation in thyroid size as a factor influencing results, and the third would also tend to remove inadvertent variations in P^{32} dosage as a factor in influencing results. On the other hand, both (2) and (3) involve several measurements: (2) uses two, (3) uses four, and since a degree of error attaches to measurements of small weights and to counts of radioactivity, it is possible that (2) or (3), or both, might be a *less* precise index of P^{32} uptake than is (1).

This matter was investigated by analyzing 7 experiments in terms of all three indices of response (see Table 1). This led to the finding that (3) was much the worst, and that (2) was somewhat inferior to (1). This conclusion is based on the ratio $L = \hat{s}/S$ where $\hat{s}$ is the range estimate of the standard deviation (Dixon and Massey, 1948) and S is the slope of the best eye-fit straight line. Small values indicate high precision. In fact, the statistic L

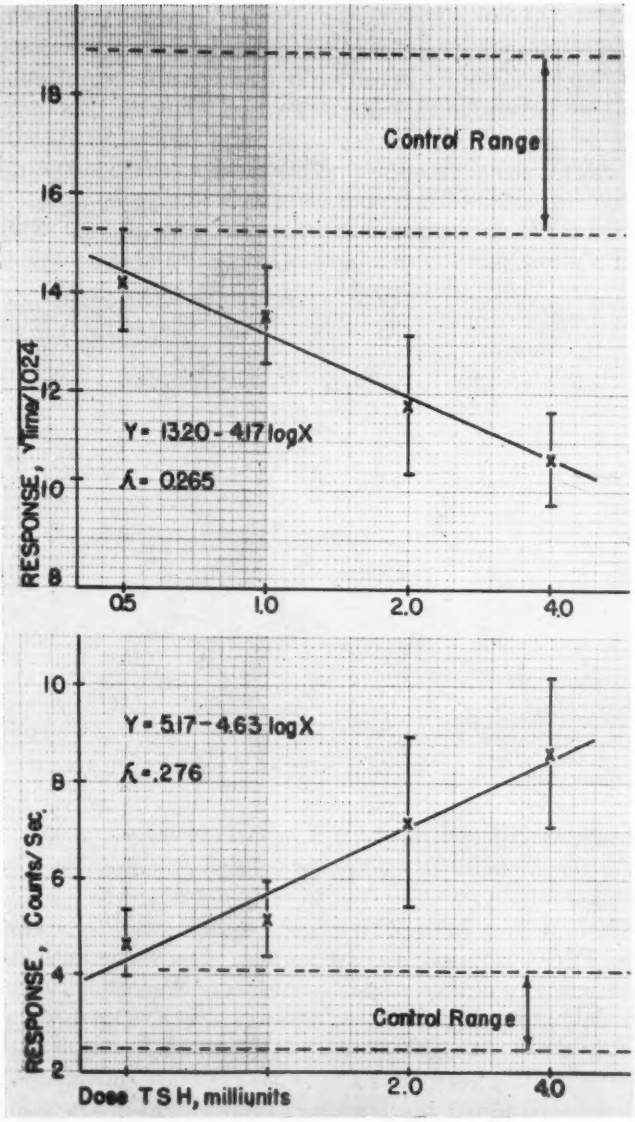

FIG. 1. The bioassay of TSH utilizing P^{32} uptake by the chick thyroid. A. (Upper Graph) Data plotted as square root of T, where T is the time to count 1024 cts. (ordinate) against log dose TSH (abscissa). B. (Lower Graph) Data plotted as counts per second per thyroid (ordinate) against log dose TSH (abscissa).

is, except for a constant multiple, an estimate of λ, the familiar index of precision.

Since (1), counts per second per thyroid, was both a more sensitive index of response and a much more convenient one to obtain (all weighing was eliminated) we have used increase in thyroid P^{32} as the sole index of TSH effect.

The actual quantity measured on each chick, then, is the time, T, to record 1024 counts. No correction was made for background activity which was very small. If the response was expressed simply as 1024/T, or as counts per second per thyroid, the variability increased systematically with the dosage. This is illustrated in Figure 1-B. These data were obtained from an experiment in which 4 groups of 9 chicks each received increasing

TABLE 1. THE VARIABILITY OF THE RESPONSE OF P^{32} UPTAKE OF CHICK THYROID TO TSH WHEN EXPRESSED AS THYROID ACTIVITY/BLOOD ACTIVITY, THYROID CTS./SEC./MG. OR THYROID CTS. PER SEC.

No. dose levels	No. chicks per dose	Thyroid activity/ blood activity[1]			Thyroid, cts./sec./mg.			Thyroid, cts./sec.		
		$\hat{\sigma}$[2]	Slope[3]	L[4]	$\hat{\sigma}$	Slope	L	$\hat{\sigma}$	Slope	L
3	9	1.81	6.0	.33	2.01	8.33	*.24*	2.92	11.67	.25
2	7	1.49	0.67	2.23	3.32	3.33	.99	4.24	4.33	*.98*
4	8	1.62	2.0	.81	2.92	2.67	1.09	3.09	6.0	*.51*
4	8	1.51	1.67	.91	2.25	4.67	*.48*	2.11	4.33	.49
3	10	1.05	3.0	.35	1.80	6.0	.33	1.77	7.33	*.24*
4	8	0.98	3.67	.27	2.09	8.33	.25	1.65	7.33	*.22*
4	8	1.90	1.67	1.14	2.27	4.33	.53	3.07	7.33	*.42*

[1] Thyroid activity = cts./sec./mg. thyroid. Blood activity = cts./sec./mg. blood.
[2] $\hat{\sigma}$ = Estimate of standard deviation obtained from range of response.
[3] Slope of best eye-fit straight dose response line.
[4] L = An approximation of λ (see text). The smallest L (indicating the highest precision) for each experiment is italicized.

doses of standard TSH. When the metameter cts./sec. is used as the index of response, the increase in variability with increasing doses of TSH is clearly demonstrable.

For all the data in a series of 10 experiments in which only known doses of standard TSH were used, several response metameters were evaluated in regard to linearity of dose response curve and homogeneity of variance. Candidates included 1024/T, T, log T, $\sqrt{T}$, $1/\sqrt{T}$. The index which appeared best in these 10 experiments was $\sqrt{T}$, which has since been used in all subsequent work. This index has a linear regression on log dose over the range of doses used in our investigations, and displays homogeneous variance. This is illustrated in Figure 1-A, where the same data in Figure 1-B are plotted using the metameter $\sqrt{T}$ as the index of response.

2. *Site and Technique of TSH Administration*

Previous workers using the chick for the bioassay of TSH administered the hormone either subcutaneously or intraperitoneally. In our initial stud-

ies we found considerable variability and lack of sensitivity in the response to intraperitoneal administration of TSH. The minimal detectable dose of TSH after intraperitoneal administration was 5 milliunits and the λ for a typical assay curve was about 0.8. In an attempt to reduce the variability, the TSH was injected directly into the blood stream of the chick by cardiac puncture. The chick heart is located just under the sternum and can easily be entered by directing the needle caudally through the suprasternal notch and withdrawing gently until blood flows freely into the needle (Figure 2). A volume of 0.1 cc. is injected. The chicks tolerate intracardiac injection surprisingly well; the mortality in a large series of intra

FIG. 2. The technique of intracardiac injection in the chick.

cardiac injections is less than 2%. Following intracardiac injection, a dose of 0.5 milliunit is easily detected, and the λ for the assay is about 0.265 (Figure 1-A). This represents a 10-fold increase in sensitivity over the technique of intraperitoneal injection and a considerable improvement in the variability.

Subcutaneous and intracardiac administration of TSH were compared using groups of 5 chicks at 5 dose levels for each method of injection. Table 2 displays the mean response and the standard deviation for each group. Study of the table indicates that the response is more marked overall with intracardiac injection. Although the control means are roughly equal the average response is substantially more marked (at each dose level) for the chicks receiving TSH by intracardiac injection. Unfortunately, conventional methods of comparison which require parallel straight dose response curves are here inapplicable, but a more elementary analysis tells much about threshold dosage. A combined estimate of the variance based on 40 degrees of freedom can be computed. Its value is 1.31. It can be deduced

TABLE 2. COMPARISON OF THE P^{32} UPTAKE RESPONSE TO TSH INJECTED SUBCUTANEOUSLY AND INTRACARDIALLY

Dose TSH milliunits	Subcutaneous injection Mean response ($\sqrt{T}$) ±standard deviation[1]	Intracardiac injection Mean response ($\sqrt{T}$) ±standard deviation[1]
0 (Saline)	17.28±1.10	16.25±1.60
0.5	15.85±1.15	13.52±0.65
1.5	17.24±0.47	12.98±1.31
4.5	14.17±1.00	11.38±1.23
13.5	12.60±0.69	11.63±1.62

[1] Five chicks per group.

from this that the standard error of a difference between any two means is 1.44. Thus, at each dose 0.5, 1.5, 4.5, and 13.5 milliunits, the intracardiac data give statistically significant (0.01 level) evidence of response to TSH administered by the intracardiac route. On the other hand, clear evidence of response to TSH given subcutaneously is first noted at a dose of 4.5 milliunits. From this we infer that intracardiac injection has increased the sensitivity of the assay considerably.

3. *Timing of the Injections*

A 6-hour time interval between intracardiac injection of TSH and time of sacrifice was found to be convenient and satisfactory. At shorter time intervals maximum P^{32} uptake could not be obtained. The time of administration of P^{32} did not seem to make much difference as long as it was constant for each experiment. The P^{32} was therefore administered intraperitoneally at the time that TSH was injected intracardially.

4. *Fractionation of Chick Thyroid P^{32}*

In order to determine if one fraction of thyroid P^{32} might vary more directly with dose of TSH, 3 experiments were done in which thyroid P^{32} activity was fractionated into acid soluble P^{32}, and each fraction was compared with total P^{32} activity. In these experiments the right lobes of the thyroids of groups of 5 chicks were pooled, homogenized in cold 10% trichloracetic acid, and the activity of the supernatant containing acid soluble phosphorus, the residue containing acid insoluble phosphorus, was compared with total activity of the pooled dried left lobes of the thyroid from the same groups of animals. Most of the activity was found to be in the acid soluble fraction, which paralleled the activity of the whole glands. No advantage to this fractionation procedure was apparent.

5. *Specificity of the P^{32} Uptake Reaction*

Previous studies have indicated that the P^{32} uptake reaction is apparently specific for TSH. Other pituitary hormones, chorionic gonadotrophins, and various proteins have failed to elicit the response (Crooke and Matthews, 1953; Lamberg, 1953). We have tested the following preparations

at a single dose level: hog Follicle Stimulating Hormone, sheep Luteotrophin, Prolactin, ACTH, Equine Anterior Pituitary Extract (Table 3-A); Pitressin®, Pitocin®, Histamine, Epinephrine, and Thyroxine (Table 3-B). None of the hormones had a significant effect on the P^{32} uptake except the preparation of LH, which was known to be contaminated with TSH. Thyroxine, itself inactive, did not interfere with the effect of TSH administered simultaneously (Table 3-B).

Potassium Iodide, in a dose of 0.005 mg., had no effect on P^{32} uptake and did not interfere with TSH administered separately immediately after the

TABLE 3. EFFECT OF CERTAIN SUBSTANCES ON THE P^{32} UPTAKE OF CHICK THYROID

Substance and dose	Mean response ($\sqrt{T}$) and standard deviation[1]
Experiment A	
FSH (Armour #264151x), 0.02 mg.	14.96 ± 1.76
LH[2] (Armour #22780), 0.02 mg.	8.16 ± 0.98
Prolactin (Armour #491154), 0.02 mg.	14.66 ± 1.11
ACTH (Pacific Lab. #B0201), 0.1 U.S.P. units	13.96 ± 0.94
Equine Ant. Pit. Extract (Victory Packing Co., Exla), 0.02 mg.	12.87 ± 1.00
TSH (Armour #491075), 0.01 U.S.P. units	7.58 ± 0.13
Controls	13.74 ± 0.87
Experiment C	
Potassium Iodide, 0.005 mg.	10.75 ± 2.06
Potassium Iodide, 0.005 mg. plus TSH, 0.0045 U.S.P. units	7.38 ± 0.89
TSH, 0.0045 U.S.P. units	7.36 ± 0.32
Controls	12.15 ± 2.08
Experiment B	
Pitressin, 0.02 U.S.P. units	13.29 ± 2.98
Pitocin, 0.01 U.S.P. units	12.18 ± 1.92
Histamine, 0.00055 mg.	13.31 ± 1.01
Epinephrine, 0.002 mg.	12.24 ± 0.81
Thyroxine, 0.005 mg.	12.47 ± 1.46
Thyroxine, 0.005 mg. plus TSH, 0.005 U.S.P. units (2 injections)	7.91 ± 1.18
TSH, 0.005 U.S.P. units	8.20 ± 0.47
Controls	13.56 ± 2.98
Experiment D	
Potassium Thiocyanate, 5 mg.	12.84 ± 0.50
Thiourea, 2 mg.	11.92 ± 0.71
TSH, 0.0045 U.S.P. units	7.58 ± 0.50
Controls	12.00 ± 1.50

[1] Five chicks used in each assay
[2] Preparation of LH known to be contaminated with TSH.

KI (Table 3-C). Potassium Thiocyanate and Thiourea, examined because of their known goitrogenic action, had no effect on the P^{32} uptake in this 6 hour assay (Table 3-D).

The most highly purified specimen of growth hormone tested in a dose of 100 μg. produced a thyroid P^{32} response equivalent to about 1 milliunit of TSH.[6] This response was compatible with the degree of TSH contamination known to be present by assay in hypophysectomized rats.

Thus far, cobaltous chloride is the only substance other than TSH which has been found to increase the P^{32} uptake of the chick thyroid. This substance was tested because of observations which indicated that cobalt sup-

[6] The growth hormone was kindly supplied by Dr. C. H. Li.

presses thyroid function in man (Kriss, *et al.*, 1955). A dose of about 2 mg. of cobalt ion per chick is necessary to produce this effect. Further observations of the cobalt-induced reaction will be the subject of a separate report.

6. *Statistical Methods*

There are standard statistical methods for comparing the relative potency of two preparations of the same drug where average response is a linear function of the log dose and the variance is homogeneous (Emmens, 1948). Experience over a period of 7 months involving 19 fifty-chick experiments in which 3 groups of 5 chicks each received pure TSH in various doses indicates that the methods used give a straight line relation over the range 0.00025 to 0.005 U.S.P. units of TSH. There is a slight seasonal variation as mentioned above. The pooled value of λ for these experiments was estimated as 0.34.

III. APPLICATION OF THE BIOASSAY METHOD

1. *The Bioassay of Purified TSH*

One application of this method of TSH bioassay is in the assay of relatively pure TSH preparations. This can be done with small groups of animals with a fair degree of precision. As an illustration of the usefulness of this technique applied to the assay of pure TSH solutions, we compared one standard TSH preparation, Armour M2105, with a different TSH preparation, Armour R491075. The preparations were administered to groups of 5 chicks at 3 dose levels each. The results are presented in Table 4. Statistical analysis of the data reveals the following: (a) The slopes of each dose-response curve do not differ significantly from one another; (b) The variances are not clearly homogeneous, but a pooled estimate of the variance was computed and found to be: S = 1.19; (c) The calculation of

TABLE 4. BIOASSAY OF PURIFIED TSH PREPARATIONS

Dose (Milliunits)	Armour #M2105		Armour #R491075	
	Response ($\sqrt{T}$)	Mean ±Std. Dev.	Response ($\sqrt{T}$)	Mean ±Std. Dev.
0.5	12.17, 9.28 13.12, 9.95 10.68	11.04 ±1.58	11.49, 9.70 10.15, 12.57 10.82	10.95 ±1.12
1.5	9.22, 8.43 8.06, 9.70 8.37	8.76 ±0.76	8.37, 9.38 10.49, 8.72 6.25	8.64 ±1.58
4.5	8.00, 7.00 7.88, 7.14 8.13	7.63 ±0.16	8.54, 6.93 5.00, 6.25 7.62	6.87 ±1.32

Calculations: Standard Deviation 1.19. *Lambda* 0.303.
Relative Potency #R491075 v. M2105 = 0.828 (0.828 units R491075 = 1 unit M2105).
Fiducial Limits of Relative Potency (95% Confidence Limit) = 0.5 to 1.38.

relative potency revealed that preparation R491075 was 0.83 times as potent as M2105. The fiducial limits of relative potency (95% confidence interval) ranged from 0.5 to 1.38.

2. *The Bioassay of TSH in Body Fluids*

Preliminary work has indicated that the residue obtained by ultrafiltration of 24-hour urine specimens from normal persons have measurable TSH activity and that in certain states of thyroid dysfunction these residues are potent in high dilutions. The slope of the response curve is consistently lower than the slope of the standard TSH curve so that direct comparisons are not possible. Studies are in progress to determine the mechanism of this altered response.

IV. COMMENT

The method here described has several advantages over the existing methods. The total assay period is 6 hours and it is possible to complete an assay within a single work day. It requires one intracardiac and one intraperitoneal injection, one dissection, and one count for each chick thyroid. Although there is species variation in sensitivity of cockerels, they are usually easily obtained, inexpensive, require no particular preparation or care, and only 15 chicks are required per assay substance. The method can detect quantities of TSH as little as 0.00025 U.S.P. units of TSH. This is not as sensitive as the histological methods in the tadpole (d'Angelo and Gordon, 1950), or in the guinea pig (Tala, 1952), but it is comparable to the I^{131} discharge methods of Gilliland and Strudwick (1953) and Adams and Purves (1955). It is much more sensitive than the I^{131} uptake methods in mice (Querido, *et al.*, 1953) or in rats (Ghosh, *et al.*, 1951). The method appears to be sensitive enough to detect TSH-like activity in urine extracts from normal persons. The method has a fair degree of precision, indicated by the pooled λ of 0.34.

SUMMARY

A method has been developed for the bioassay of Thyrotropic Hormone (TSH) which utilizes the uptake of P^{32} by the thyroid of the one day old cockerel 6 hours after the intracardiac injection of TSH. Statistical analysis indicates that the response metameter, the square root of T, where T equals the time to count 1024 counts per thyroid, yields both linearity of response and homogeneous variance. Standard statistical analysis can therefore be applied to the bioassay of purified TSH in the dose range of 0.00025 to 0.005 U.S.P. units of TSH with a degree of precision indicated by a pooled λ of 0.34. Preliminary studies have indicated the presence of modified TSH activity in the residue of an ultrafiltrate of normal human urine.

ADDENDUM

Since the preparation of this manuscript, Levey, *et al.*, have reported a method for the bioassay of TSH (*Endocrinology 58:* 420, 1956) based on the I^{131} uptake of the thyroid powder prefed rat, which has about the same sensitivity but a longer dose response range than the chick P^{32} method. These authors also noted that certain materials of biological origin yield a flattened dose response curve. This is comparable to our observation on human urine extracts and suggests that these substances are not identical to purified TSH.

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STEROID-INDUCED ADRENAL-PITUITARY HYPOFUNCTION

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A STANDARDIZED procedure utilizing potassium chloride as a stressing agent has been developed to demonstrate steroid-induced adrenal-pituitary axis hypofunction (Collins, 1955a). The data to be presented indicate that the decreased resistance of the steroid-treated animal is the result of an inadequate response of the pituitary gland to the stress and not adrenal hypofunction *per se*.

METHODS

Male albino rats of the Upjohn colony weighing 175–200 gm. and fed Archer dog pellets *ad libitum* were used throughout the experiment.

Group I. The animals were injected subcutaneously once daily with 4 mg. hydrocortisone (F) alone or in combination with varying doses of ACTH (Upjohn). Testosterone propionate (T.P.) was administered concurrently with F but injected in an area removed from the site of F injection. On the evening of the 10th day, food but not water was removed from the cages. Following the 18-hour fast, stress was induced by intraperitoneal (I.P.) injection of 0.16 M KCl, 5 cc. per 100 gm. body weight.

Group II. Following a 10-day treatment with 4 mg. F, the animals were treated in one of the following ways.

a. Animals were injected subcutaneously on the afternoon of the 10th day (8 hours after last F injection) and again 17 hours later (1 hour prior to stress) with ACTH in 16% gelatin, *Lipo-Adrenal Cortex*® (LAC), desoxycorticosterone acetate (DCA) in cottonseed oil, or corticosterone (compound B) suspended in 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol and 0.9% sodium chloride.

b. Twenty-four hours following the last F injection the animals were anesthetized with ether and then given an infusion, into the tail vein, of one of the following preparations: 4 mg. F hemisuccinate sodium, 4 U. ACTH (Upjohn or Armour), 4 mg. desoxycorticosterone glucoside (DCG) or aldosterone,[1] 5 or 10 μg. As soon as the animals became conscious they were subjected to the potassium stress.

RESULTS

Table 1 demonstrates that concurrent administration of T.P. or ACTH with F is without effect in maintaining the functional integrity of the adrenal-pituitary axis. It can be seen that 7% of the T.P.-F and 10–20% of the ACTH-F injected animals survived whereas 80–90% of the non-F treated animals were able to tolerate the potassium stress.

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[1] Aldosterone was obtained from R. E. Knauff, Department of Biochemistry, The Upjohn Company.

TABLE 1. EFFECTS OF POTASSIUM STRESS TO ANIMALS FOLLOWING INJECTIONS OF HYDROCORTISONE ALONE OR IN COMBINATION WITH ACTH OR T.P.

No. rats	Body wt., gm.	Treatment 1×/day/10 days	Survivals %
39	190	Untreated	90
40	162	4 mg. F	14
20	169	4 mg. F+1 U. ACTH	15
10	156	4 mg. F+2 U. ACTH	10
10	163	4 mg. F+3 U. ACTH	20
10	152	4 mg. F+4 U. ACTH	20
15	231	4 U. ACTH	79
15	170	4 mg. F+2 mg. T.P.	7
15	228	2 mg. T.P.	93

Prophylactic fore-treatment with various adrenal preparations to hypofunctional rats afforded minimal protection in that 33–40% of the animals so treated compared with 12% of the non-treated animals survived the potassium stress (Table 2).

Intravenous administration of aldosterone or ACTH was effective in

TABLE 2. PROTECTION AFFORDED BY ADRENAL PREPARATIONS AGAINST POTASSIUM STRESS TO HYDROCORTISONE-TREATED ANIMALS

No. rats	Body wt., gm.	Post-hydrocortisone treatment*	Survivals %
19	Controls		90
55	170	None	16
15	172	2 mg. DCA	33
15	162	5 mg. cpd. B	40
15	170	5 U. LAC	37
15	170	5 U. ACTH	13

* Injections 18 hours and 1 hour prior to stress.

giving protection in 60–90% of the animals. The F hemisuccinate sodium and DCG were both ineffective at the doses used (Table 3).

DISCUSSION

Exogenous adrenal cortical hormones cause a suppression of ACTH release from the pituitary, thereby causing atrophy of the adrenal cortex

TABLE 3. PROTECTION AFFORDED BY ADRENAL PREPARATIONS ADMINISTERED INTRAVENOUSLY AGAINST POTASSIUM STRESS TO HYDROCORTISONE-TREATED ANIMALS

No. rats	Body wt., gm.	I.V. treatment	Survivals %
20	163	None	25
10	167	4 mg. F hemisuccinate sodium	30
10	171	4 mg. DCG	30
20	171	4 U. ACTH (Lot #10438, 10438-1-2)	85
29	158	4 U ACTH (Lot #10801)	90
15	160	4 U. ACTHAR Gel (Armour)	87
5	162	5 μg. Aldosterone	40
10	171	10 μg. Aldosterone	60

(Ingle, 1938). This steroid-induced atrophy can be prevented by simultaneous injections of ACTH (Stafford) or by testosterone (Winter *et al.*, 1951; Gaunt *et al.*, 1953). Although adrenal structure is maintained by these preparations, the data presented here indicate that adrenal secretory activity is still hypofunctional when the treated animal is subjected to potassium stress.

During steroid therapy adrenal cellular activity of man is functionally arrested and is not immediately responsive to exogenous ACTH (Salassa, 1953; Engleman, 1953). The steroid-induced hypofunction is also true of the dog but it can be prevented by the simultaneous administration of ACTH (Collins, 1954). This cellular deficiency apparently does not occur to the same degree in the rat, for the protection following infusion indicates: (1) the adrenal of the F-treated rat, atrophied though it be, still is capable of responding (with rapidity) to the intravenous administration of ACTH. This has not been demonstrated in the human or the dog (Engleman, 1953; Collins, 1954). (2) During stress the adrenal cells are not secreting hormone due to inadequate pituitary stimulation, as demonstrated in those animals whose adrenal structure was maintained by simultaneous administration of ACTH or testosterone. (3) The absence of adequate ACTH secretion suggests that the exogenous hydrocortisone has suppressed pituitary function. Farrell and Laqueur (1955) have demonstrated that cortisone decreases the ACTH content of dog pituitaries and their results indicate that the exogenous steroid inhibits synthesis as well as release of ACTH.

Since we do not know how steroids are utilized, the lack of response to the adrenal preparations other than aldosterone cannot be explained at this time. However, Sydnor (1955) has demonstrated that hydrocortisone is effective in inhibiting ACTH discharge under conditions in which the rate of release should be maximal.

Although final proof is lacking at the present time, several possible explanations can be offered for the response to aldosterone while other adrenal preparations were more limited in their effects. The mineralocorticoid activity of aldosterone could have an influence in regulating any fluid and electrolyte shifts necessary to handle the injected potassium solution. Secondly, the glucocorticoid activity attributed to this steroid could help to maintain the functional integrity of the peripheral vasculature during the period of stress. The limited response following DCA or DCG as well as the unaltered resistance to the stress in sodium-depleted animals (Collins, 1955b) suggest that the effect of potassium is not only an "electrolyte stress" but an organic disturbance as well.

SUMMARY

Exogenous hydrocortisone in the rat renders the pituitary rather than the adrenal hypofunctional. Although the adrenal gland is atrophied, func-

tional integrity of the cells appear to be maintained. It has been demonstrated that the atrophied adrenal gland can respond with rapidity to the intravenous administration of ACTH. Aldosterone as well as ACTH, when given by vein, corrects the decreased tolerance to potassium chloride stress in the hydrocortisone-induced hypofunctional animal.

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ANDROGENIC AND MYOTROPHIC PROPERTIES OF ORALLY ADMINISTERED 9-FLUORO-11-OXY-METHYLTESTOSTERONES

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THE oral efficacy of steroid hormones and hormone-like compounds varies substantially from hormone to hormone and from species to species. Most steroid estrogens are far more effective parenterally than orally (Segaloff, 1949). Adrenocortical hormones of the 11-oxygenated type are highly effective by the oral route (Kuizenga, Nelson, and Cartland, 1940; Freyberg *et al.*, 1950), but desoxycorticosterone is almost ineffective orally (Kuizenga, Nelson, and Cartland, 1940). Progesterone is orally active, but not nearly as potent as when given by injection (Bickers, 1949). Testosterone and its esters are not given orally in man because of low potency by this route, but methyltestosterone is orally effective (Turner, 1950). The factors which can influence oral effectiveness of steroids are concerned with gastrointestinal inactivation and absorption as well as with rates of liver and peripheral tissue degradation and conjugation.

To date the most successful chemical modification of testosterone for oral use has been 17α-methyl substitution. This report presents data which show that the introduction of fluorine at C-9 and/or oxygen at C-11 are effective means of enhancing oral potency of methyltestosterone in the rat.

METHODS AND MATERIALS

Steroids. The compound used in this study (Herr, Hogg, and Levin, 1955) were 17β-hydroxy-17α-methyl-4-androstene-3-one (methyltestosterone, MT), 11β,17β-dihydroxy-17α-methyl-4-androstene-3-one (hydroxy-MT), 17β-hydroxy-17α-methyl-4-androstene-3,11-dione (keto-MT), 9α-fluoro-11β,17β-dihydroxy-17α-methyl-4-androstene-3-one (F-hydroxy-MT), and 9α-fluoro-17β-hydroxy-17α-methyl-4-androstene-3,11-dione (F-keto MT).

The steroids were given to rats in the form of cottonseed oil solutions or suspensions, the physical state depending on concentration. The crystalline steroids were dissolved in acetone, an appropriate quantity of cottonseed oil was added, and acetone was removed by heating and passing air through the mixture. Suspensions, when obtained, were of very finely divided crystals.

Animals. Male rats of the Upjohn-Sprague-Dawley colony were castrated at 26–27

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days of age and were fed Archer dog pellets and tap water *ad libitum* throughout the experiment. The beginning weight of these animals was 74 gm. ±5.

Test Regime. The seminal vesicle of the castrate rat has long been used as an indicator of the "androgenic" action of steroids; the growth of the levator ani muscle in the same animal is considered by some investigators to be an index of the protein anabolic effect of the steroids (Eisenberg and Gordan, 1950; Hershberger, Shipley, and Meyer, 1953; Barnes, Stafford, Guild, Thole, and Olson, 1954). These indices were used in the experiments reported here.

Beginning on the day following castration and continuing for 9 days, the animals were dosed once daily with 0.2 to 0.4 ml. of the appropriate steroid preparations by insertion of a specially blunted 18-gauge needle into the esophagus. On the day following the last dose the rats were sacrificed and the seminal vesicles (exclusive of coagulating gland and contained secretion) and the levator ani muscles were weighed on a Roller-Smith balance.

Experiments were designed as follows: Ten rats constituted a dosage group. Three doses of the standard compound, MT, were tested simultaneously with three doses of unknown compound. The daily doses of methyltestosterone were always 1, 2, and 4 mg., but the doses of unknown compounds had to be adjusted according to potency, as the reliability of the assay depends upon approximately equal levels of response with standard and unknown. Four such assays were conducted with hydroxy-MT, F-hydroxy-MT, and F-keto-MT. The data on keto-MT are more limited, as only two single-dose experiments were performed and the values were interpreted from a previously prepared methyltestosterone dose-response curve rather than from the responses of simultaneously-treated standard animals.

Potency ratios between standard and unknown were calculated by the Irwin (1937) method. Confidence limits were not calculated, as it is our opinion that replication of the assay affords more assurance of reliability than such statistical manipulation.

RESULTS

Table 1 presents the results of a typical assay of hydroxy-MT. The calculation of potency ratios in this particular assay showed that hydroxy-MT was 83% of MT in capacity to stimulate the seminal vesicle (androgenic) and 278% of MT in capacity to stimulate the levator ani (myotrophic). Even without the calculation it is readily apparent, from scanning the

TABLE 1. TYPICAL ASSAY COMPARING 11β-HYDROXYMETHYLTESTOSTERONE WITH METHYLTESTOSTERONE

Test substance	Dose (mg./rat/day)	No. rats	Body weight		Seminal vesicle weight (mg.)*	Levator ani weight (mg.)*
			Initial	Terminal		
Vehicle only	—	26	74	111	9 (6–11)	22 (17–33)
Methyltestosterone	1	10	73	125	24 (13–35)	46 (34–56)
	2	10	69	113	44 (31–62)	50 (43–57)
	4	10	73	127	84 (71–102)	76 (62–101)
Hydroxy-MT†	1	10	79	135	24 (15–30)	64 (55–81)
	2	10	74	121	44 (26–75)	82 (68–95)
	4	10	75	132	65 (52–91)	91 (80–99)

* Figures in parentheses indicate ranges of organ weights.
† Irwin calculation on this assay shows hydroxy-MT to be 2.78 × methyltestosterone as a myotrophic agent, 0.83 × methyltestosterone as an androgen.

TABLE 2. SUMMARY OF ALL ASSAYS ON ALL COMPOUNDS

Compound	No. assays	No. rats	Potency (×methyltestosterone)*			
			Androgenic		Myotrophic	
Hydroxy-MT	4	240	0.9	(0.7– 1.1)	3.0	(1.65– 4.62)
F-Hydroxy-MT	4	240	9.5	(9.0–10.0)	20.0	(18.0 –22.0)
Keto-MT	2†	10	0.65	(0.6– 0.7)	1.4	(1.25– 1.60)
F-keto-MT	4	240	8.5	(7.0–10.0)	22.0	(13.6 –31.0)

* Figures in parentheses indicate ranges of potency ratios, unknown/methyltestosterone.
† Only two single-dose assays on this compound. Potency ratios estimated by comparison of results with standard dose-response curve for methyltestosterone.

data and interpolating, that these quantitative relationships exist; e.g., the dose of hydroxy-MT which produced an 82-mg. levator ani was only half the dose of MT which produced a 76-mg. levator ani.

Table 2 summarizes all the assay information obtained on the four new compounds. On the androgenic side, keto-MT is somewhat less active

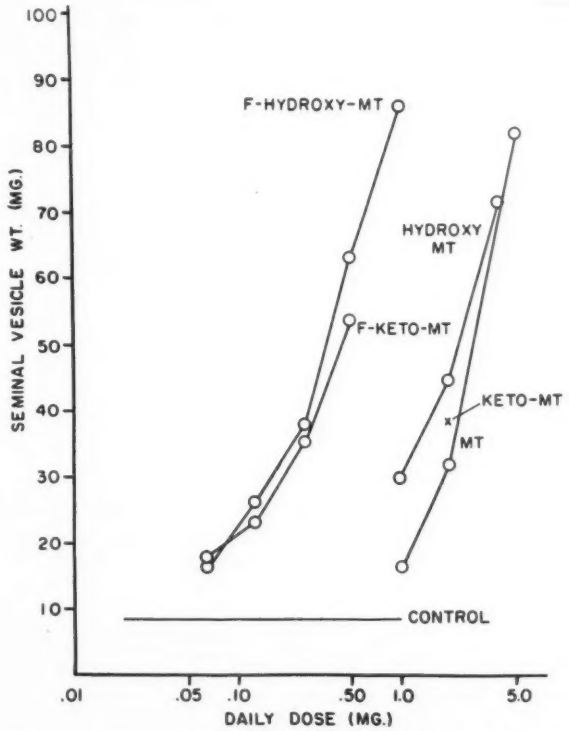

FIG. 1. Graph summarizing all data on relative androgenic potencies of the five compounds studied. Each point is the mean value of the seminal vesicle weights of forty rats except that showing "keto-MT" which is the mean of ten animals.

than MT, hydroxy-MT is about as active as MT, while F-hydroxy-MT and F-keto-MT are about ten and eight times MT respectively. Throughout the series there is an element of preferential myotrophic activity, the levator ani stimulation being two to three times as great as the seminal vesicle activity.

Figures 1 and 2 show in graphical form means of all organ weights obtained in this series of experiments.

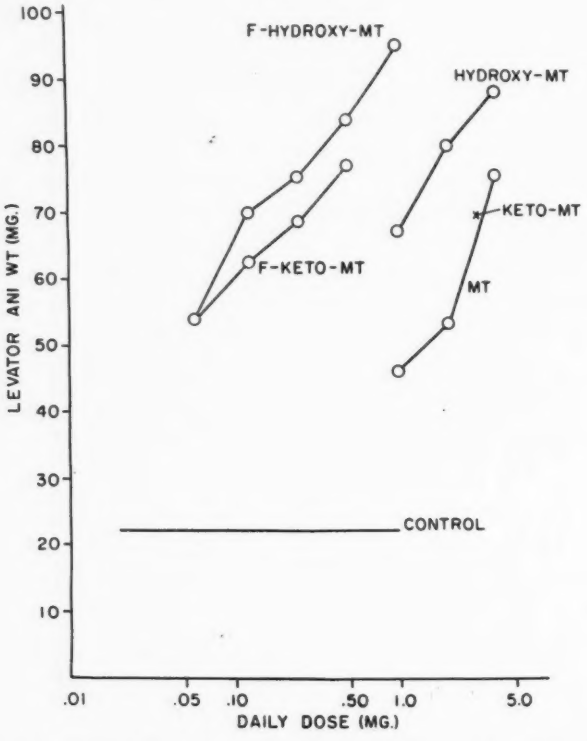

FIG. 2. Graph summarizing all data on relative myotrophic potencies of the five compounds studied. Each point is the mean levator ani weight from the same groups of animals whose seminal vesicle weights are shown in Fig. 1.

DISCUSSION

There is no striking difference between the seminal-vesicle-stimulating properties of MT, keto-MT, and hydroxy-MT. The levator-ani-stimulating activity in the 11-oxygenated compounds is definitely greater than that of MT. Hydroxy-MT, for example, is three times as potent as MT as a myotrophic agent. This is very interesting when considered in connection with the observation of Jailer *et al.* (1955) that the somatic development

of congenital adrenal virilism patients is frequently advanced beyond the development of body hirsutism ordinarily associated with testosterone treatment, and that the urine of such patients is rich in 11-oxygenated 17-ketosteroids.

The high activity of the 9α-fluoro compounds reported here is a stimulating finding for students of structure-function relations. Substitution of fluorine for hydrogen at C-9 has brought about remarkable changes in activity of a number of active adrenocorticoids and related compounds in the pregnane series (Fried and Sabo, 1953; Borman, Singer, and Numerof, 1954). This report shows that C-9 fluorine also enhances the activity of two 11-oxygenated androstane compounds, a surprising finding in view of the probable difference in metabolic pathways of the two types of steroid suggested by their different biological properties.

SUMMARY

The weights of the seminal vesicle and levator ani muscles of castrate immature rats have been used as end-points to assay the "androgenic" and "myotrophic" activity of four analogues of methyltestosterone.

11-Keto-methyltestosterone is slightly less potent than methyltestosterone as an androgen and slightly more active as a myotrophic agent. 11β-Hydroxy-methyltestosterone is 0.9 times as potent as methyltestosterone as an androgen and 3 times as potent as a myotrophic agent. 9-Fluoro-11β-hydroxy-methyltestosterone is 9.5 times as potent as methyltestosterone as an androgen and 20 times as potent as a myotrophic agent. 9-Fluoro-11-keto-methyltestosterone is 8.5 times and 22 times methyltestosterone by the same measures of potency.

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FUNCTIONAL CORPORA LUTEA MAINTAINED FOR MONTHS BY AUTOGRAFTS OF RAT HYPOPHYSES[1]

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THE rat hypophysis, when isolated from the diencephalon by transplantation to the kidney, can nevertheless liberate luteotrophin during the following week in sufficient amount to maintain luteal function (Desclin, 1950). Desclin's rats having been implanted in advance with pellets of stilbestrol, the liberation of luteotrophin was interpreted to be a direct effect of estrogen. Subsequently, however, Everett (1954) reported that functional corpora lutea are readily and consistently obtained in rats bearing hypophyseal autografts, without any stimulus other than may be inherent in transplantation itself.

The question was raised that possibly this liberation of luteotrophin represents not true secretion, but only a slow absorption of hormone already present. Various reasons were advanced, however, for discounting this possibility. Conclusive evidence is furnished by long-term experiments, some of which will now be presented in detail. It will be shown that luteotrophic function of grafted hypophyses continues without apparent diminution in the course of experiments lasting as long as 3 or 4 months. The original corpora lutea forming at the time of transplantation remain active throughout these long intervals and present no evidence of deterioration.

MATERIALS AND METHODS

The experimental animals were 24 adult virgin female rats of our inbred strain. With the exception of one 12-month animal the age was 7 to 10 months at the time of operation. Individuals selected were sound and without outward evidence of respiratory or other disease. The vaginal smear history had been recorded for several weeks, six days each week; the prediction of ovulation date in the current cycle was based on that history (cf. Everett, 1948; Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1949). Combined hypophysectomy and autotransplantation of the pars distalis was performed on the day after presumptive ovulation, at which time the vaginal smear registered full cornification (stage III).

The operation was performed under ether anesthesia, the animal having been atropinized (10 mg./kg.) to ensure a dry trachea. Surgical approach to the left kidney was first made and temporarily closed, after which the parapharyngeal approach was made to the hypophysis. After exposure of the trachea a polyethylene tube was passed into it

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through the oral cavity and larynx, to be left in place throughout the operation. Through a hole in the sphenoid drilled with a #11 dental burr, the gland was drawn by vacuum into a glass tube that fitted the opening, and was then drawn farther back into a trap by dipping the tube in sterile saline. Under observation with a dissecting microscope (7×) the hypophyseal capsule was exhausted by vacuum through a slender polyethylene tube.[2] After the neck incision had been closed the left kidney was again visualized and with fine forceps a slit was made in the renal capsule to form a pocket, usually midway along the lateral border. The hypophysis was recovered from the trap where it had been resting in saline at room temperature, the posterior lobe was discarded, and the pars distalis, either intact or as two halves, was inserted into the renal pocket. Pressure of surrounding tissues was usually sufficient to keep the gland in position. After operation

TABLE 1. GROUPING OF EXPERIMENTS ACCORDING TO TEST PROCEDURE AND VAGINAL RESPONSE TO ESTROGEN

Group	No. of animals	Duration in days[a]	Estrogen test	Vaginal response
A	6	**26**[b] **53**[b] **53**[b] 64 64 **90**	None	———
B	13	**23** **33** 33 33 50 **60** 60 60 88 **89** 90 104 104	Final week	Mucification
C	5	**94**[c] 94[c] **98**[d] **103**[d] 120[d]	Before graft removal	Mucification
			After graft removal	Cornification

[a] Figures in bold face represent animals in which no trace of pars distalis remained in the hypophyseal capsule. Others retained small fragments of doubtful significance.
[b] Dates of castration, after which the standard estrogen regime was instituted, with resulting cornification of the vagina. Autopsy was 7 days after castration.
[c] Tested with estrogen on 3 occasions before graft removal at 88 days (see text).
[d] Tested with estrogen on one occasion shortly before graft removal.

the animals were housed 2 or 3 to the cage and supplied with 5% dextrose in their drinking water. The basic diet remained unchanged (Purina dog chow *ad libitum*). Regular vaginal smears were continued.

As in the short-term experiments, there was no special stimulus to induce pseudopregnancy.

Because of the intended long duration and the attendant likelihood of impaired responsiveness of the uterus to trauma, the deciduoma reaction did not seem an appropriate test for progesterone secretion. Instead, it was decided to inject an excess of estrogen during the final week. Progesterone secretion would then be shown by vaginal mucification (Allen and Meyer, 1935; Astwood, 1941; Desclin, 1950). The standard procedure was to inject subcutaneously 50 μg. estradiol benzoate[3] on a given day, another 50 μg. 3 days later and 25 μg. after another 2 days. The usual autopsy date was 2 days after the final injection.

Duration of experiments ranged from 23 to 120 days (Table 1). In Group B, the definitive series, the ovaries and graft remained in place until the very end of the experiment, the estrogen test being given during the final week. In Group A no test was made

[2] Clay-Adams #PE 50, bent at 90° near the tip and stiffened by passage through a hypodermic needle of appropriate size.

[3] Progynon B, very generously supplied by the Schering Corporation, Bloomfield, N. J. Concentration 0.333 mg./cc. sesame oil.

before oöphorectomy or autopsy; ovaries, uteri and vaginas were thus acquired for histologic comparison with the other groups. In Group C the graft was removed after one or more estrogen tests had produced unequivocal mucification of the vagina. The date of removal was approximately 10 days after the last injection of a series. In 2 rats the entire left kidney was excised, but the other 3 grafts were easily recovered by peeling them away from the kidney surface. Another estrogen sequence was instituted 24 hours later.

At the end of each experiment the animal was dispatched with Nembutal and the graft and reproductive tract were at once examined *in situ*. Under the dissecting microscope rough approximations of corpus luteum diameters were made by using fine forceps as calipers. Ovaries, adrenals and occasional thyroids were weighed before fixation. Tissues routinely fixed in Zenker-acetic were: the graft, one or both ovaries, an adrenal, thyroid, and samples of uterus and vagina. The hypophyseal capsule with adjacent cranial floor was fixed and decalcified either by storing in Bouin's fixative for 5 or 6 weeks or by immersion in Heidenhain's Susa for 24 hr. followed by 5% trichloracetic acid for 2 to 3 days. Each sample of tissue was serially sectioned and stained by a modified Masson trichrome technique.

All sections of the hypophyseal capsule were fully searched for remnants of the gland. Although 16 of the 24 specimens retained small fragments (cf. Table 1), these were so small that they were not recognized under the dissecting microscope at autopsy. Residues of such dimensions were ineffective in control animals of the short-term series (Everett, 1954, p. 687).

OBSERVATIONS

The results were surprisingly uniform with respect to survival of the graft, status of the reproductive organs, effects of estrogen, condition of adrenals and thyroids, and general condition of the animals.

Grafts. The transplanted pars distalis remained healthy throughout even the longest experiments. At autopsy its original form was easily recognized and the color was that of the normal tissue. Vascularization was excellent, whether or not the glandular parenchyma was closely adherent to the renal cortex. Characteristically, prominent vessels passed to the graft from the body wall or mesovarium. No significant histologic differences were observed between grafts that had remained in place several months and those of the short-term series only 8 days after transplantation (Everett, 1954).

In the sections it is common to find that well-organized parenchyma with a normal pattern of open sinusoids extends inward from all surfaces for 0.1 to 0.2 mm. to a core of areolar tissue (Fig. 1, A, B). Although the material is not optimal for cytologic investigation, the impression is given that basophiles are absent and that acidophiles, especially small degranulated ones, are abundant. After estrogen treatment there are notable increases in number of cells with hypertrophied Golgi zones and in frequency of mitotic figures.

Vagina. On the day after transplantation, as after hypophysectomy alone, there was often a very pronounced sloughing of vaginal epithelium. By the second day, however, the smear was usually of the simple, anestrous type, remaining so in the absence of estrogen treatment. A small amount of mucus was sometimes recognizable after staining. Histologically the

epithelium of untreated animals was seen to be atrophic (Fig. 2, A). By contrast, the epithelium of the 3 estrogen-treated castrates of Group A was hypertrophic and fully cornified within the week after the first injection.

In the presence of both graft and ovaries (Group B) the consistent effect of estrogen was vaginal hypertrophy with mucification (Fig. 2, B). The great majority of smears remained thinly leucocytic with a sparse population of epithelial cells, a heavy discharge of mucus becoming evident within 3 days after the first injection. In one exceptional animal there intervened a proestrous smear, and in four others a cornified smear, before the vagina became distinctly mucified. In one of the four the smear remained partially cornified for another 2 days. Leucocytes then reappeared, but frank mucification was delayed until the end of the test week.

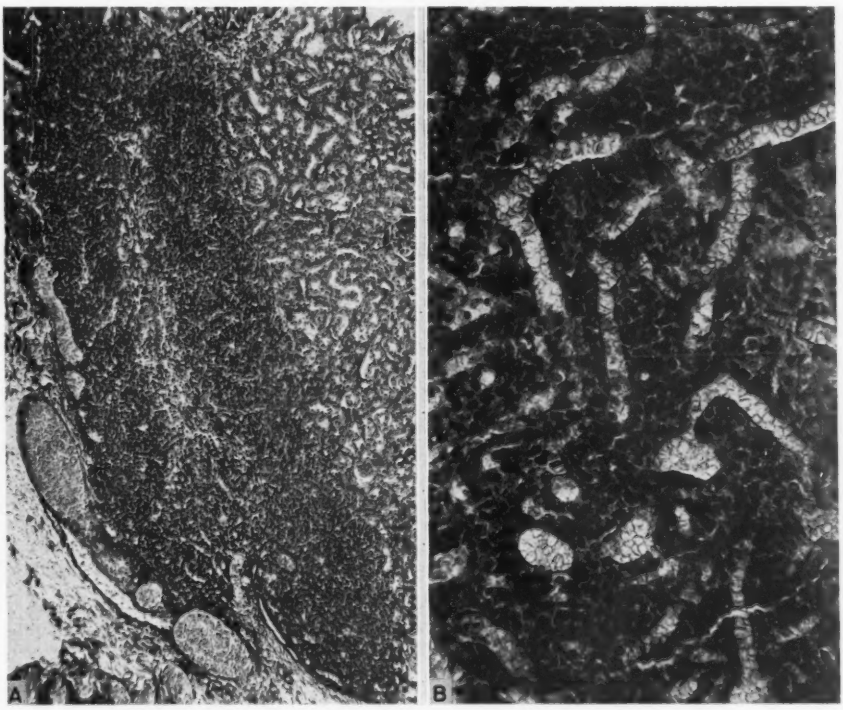

FIG. 1. Grafts of pars distalis.

A. One of the grafts of Group C, surgically removed with kidney 86 days after transplantation. The portion shown represents approximately one-third of the entire graft. An equally extensive mass extends beyond this field to the upper left and the margin of another is barely visible at the lower left. Note the lightly stained connective tissue core and the prominent veins immediately superficial to the graft. (♀ 4787.5) ×70.

B. Portion of a graft from Group A, obtained at autopsy 90 days after transplantation, showing detail of parenchyma and sinusoid pattern. (♀ 4822.1) ×210.

After removal of the grafts (Group C), repetition of the estrogen test quickly provoked persistent cornification. One week later all vaginas were histologically like that in Figure 2, C. It is thus emphatically demonstrated that vaginal mucification was dependent on the grafts.

Uterus. In the absence of estrogen (Group A) the uteri remained slender and pale. In Group B, however, they were enlarged, heavy-walled and hyperemic; lumens were enlarged, but not distended. In the 3 estrogenized castrates of Group A, the uteri became hyperemic and markedly distended. This same picture was observed at autopsy in all rats (Group C) that received estrogen during the week after graft removal. Estrogen, then, produced uterine distention if either the graft or the ovaries were absent.

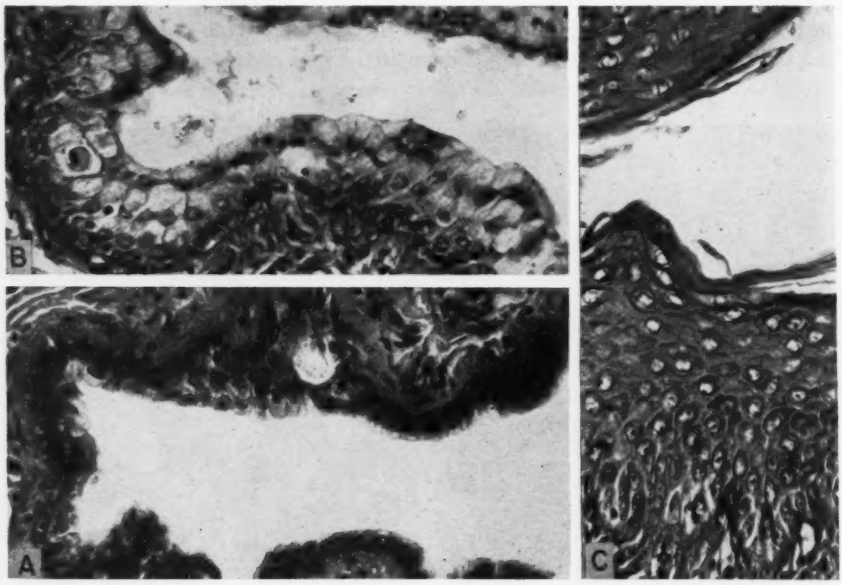

FIG. 2. Vaginal epithelium characteristic of the three experimental groups. ×290.

A. Atrophic epithelium, 90 days after pituitary transplantation, no treatment. (♀ 4822.1).

B. Mucified epithelium, 90-day experiment, 125 μg. estradiol benzoate during final week. (♀ 4685.2).

C. Cornified epithelium, 94-day experiment, graft removed at 86 days, 125 μg. estradiol benzoate during final week. Smears had been mucified in earlier tests. (♀ 4784.3).

The histology of the uteri is complex and will not be dealt with here.

Ovaries (Figs. 3 and 4). All ovaries were characterized by (1) pronounced atrophy of the follicular apparatus and the interstitial tissue, and (2) persistence of a set of large corpora lutea unless or until the graft was removed.

Few follicles, usually none at all, could be recognized under the dissecting microscope (7×). Small numbers of growing follicles were found in the sections, but few healthy follicles with antra were to be seen. The latter were restricted to 4 animals that retained small fragments of pars distalis in the hypophyseal capsule. The interstitial tissue and the small follicles that it contained were often difficult to see in the freshly excised ovary, being hidden deeply in the intervals between corpora lutea. The color was

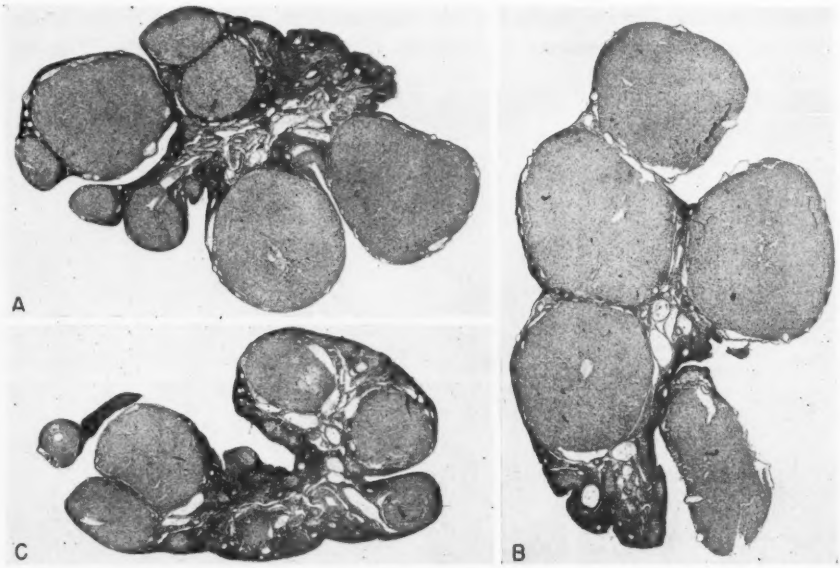

FIG. 3. Ovarian histology characteristic of the three experimental groups. Note the atrophy of interstitial tissue and follicular apparatus in all examples. ×16.

A. Untreated animal, 90 days after pituitary transplantation. Three CL_I and 4 CL_{II}. (♀ 4822.1).

B. Enlarged CL_I in a 60-day experiment after 125 μg. estradiol benzoate during final week. (♀ 4706.5).

C. Corpus luteum atrophy 8 days after removal of the graft, 94-day experiment, 125 μg. estradiol benzoate repeated during final week. Four CL_I and at lower right a CL_{II}. (♀ 4787.5).

brownish yellow like that of atrophic ovaries long after simple hypophysectomy. Histologically the interstitial cells exhibited the densely staining "wheel" nuclei and diminished cytoplasm of long-standing LH deficiency. Hence there is no evidence of either FSH or LH (ICSH) activity on the part of the graft.

The prominent corpora lutea, numbering from 7 to 13 in each animal, are believed to represent the ones that were organizing at the time of hypophyseal transplantation. They were usually accompanied by several dis-

tinctly smaller luteal bodies that are believed to represent the next youngest set (see Fig. 3, A). The two sets will occasionally be referred to as CL_I and CL_{II}, respectively. When not qualified, the expression "corpora lutea" will refer to CL_I.

In Groups A and B the corpora lutea were indistinguishable from those that one would normally find in pseudopregnancy and late pregnancy, respectively. This was true with respect to their color, texture and dimensions as seen in the freshly excised ovary, as well as to their histologic characteristics. Dimensions were notably uniform, not only within the individual animal, but among individuals of the respective groups (Fig. 3).

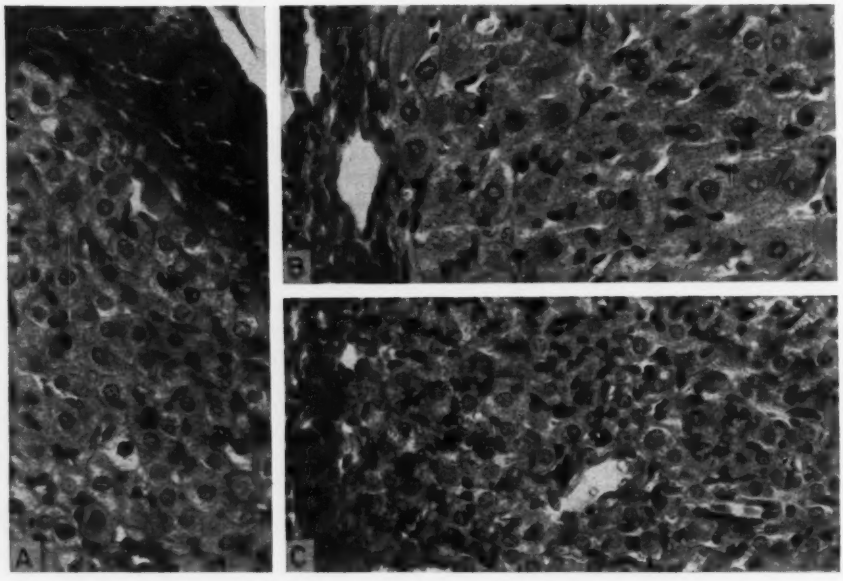

FIG. 4. Portions of corpora lutea (CL_I) from ovaries represented in Figure 3, to show characteristic differences in size of luteal cells. Same lettering. ×325.

In Group A, CL_I measured approximately 1.5 mm. in diameter before fixation, very like corpora lutea of normal pseudopregnancy or early pregnancy. In Group B in response to estrogen they were obviously much enlarged, to approximately 2 mm. In Group C, on the contrary, during the week after removal of the grafts they became reduced to 1.2 mm. or less. These characteristic sizes directly express the sizes of constituent parenchymal cells (Fig. 4). The examples illustrated could have been replaced by any of the others in the respective categories, with equally distinct contrasts. There was no effect related to elapsed time, for in either Group A or Group B the CL_I from 1-month and 3-month experiments were histologically identical.

The same was true, in fact, for CL_{II}. Photomicrographs of these elements

from the three experimental groups would show cell volumes and cytologic detail almost identical to those shown in Figure 4. A moderate relative increase in number of stromal cells was the only feature, other than small size, that distinguished CL_{II} from CL_{I}.

Ovarian weights in Group B ranged from 42 to 67 mg., averaging 54 mg. Such weights are similar to those in cyclic rats, but far short of weights that would be expected during late gestation (Donaldson, 1924), a difference that may be ascribed to atrophy of the interstitial gland and follicular apparatus. In Group A only two pairs of ovaries were weighed (47, 38 mg., resp.). In Group C the range was 14–27 mg., averaging 20 mg.

Thyroids and adrenal cortex. Both of these glands were consistently atrophic. The small numbers of individuals in the various categories (terminal experimental conditions, duration and age) do not warrant statistical analysis, however. Thyroid weights were obtained in only 6 rats (Groups A and B), in which the range was 8.1 to 12.0 mg. Adrenal weights (pairs) ranged from 9.3 to 22.3 mg., showing a downward trend with time. In Group C the adrenal weight range was 9.6 to 11.4 mg., in close similarity to that in five 3-month experiments of Groups A and B (range: 9.3 to 13.3 mg.).

Body weight and general condition. All animals represented in Table 1 remained in sound condition throughout the experiments. Within 2 days after pituitary transplantation body weights had dropped 5–10%, but thereafter remained fairly constant, subject to adequate supply of dextrose water. Among 16 rats which averaged 224 gm. before operation, the average loss after 2 months was 13 gm. (6%). Rats in Group C experienced an additional and rapid loss (7%) within the 8 days after removal of the graft. The hair remained fine and silky; once the shaven areas were again covered it would have been practically impossible to distinguish the experimental animals by outward appearance had they been mingled with normal rats.

One case of inanition caused by a jaw defect, one death (54 days) from acute respiratory distress, and a death (79 days) ascribed to inadequate survival of the graft represent the only "failures." In this last animal the pars distalis had been badly fragmented during transplantation; only a minute piece was recovered at autopsy.

DISCUSSION

Several facts together demonstrate that the transplanted pars distalis secreted luteotrophin throughout the many weeks' duration of these experiments: (1) maintenance of large corpora lutea as long as the transplant remained in place; (2) enlargement of the corpora lutea to "pregnancy size" when estrogen was administered; (3) their rapid regression after the graft had been removed; and (4) the vaginal mucification response to excess estrogen. The increase of luteal volume following estrogen injection is considered to be an expression of synergy of estrogen with luteotrophin

secreted by the gland, in as much as it has been reported by Desclin (1949) that extrinsic estrogen serves in this way to enlarge corpora lutea maintained by extrinsic lactogen. Vaginal mucification following estrogen injection is in itself strong presumptive evidence of progesterone secretion and thus, by definition, an indicator of luteotrophin activity (Astwood, 1941). Progesterone secretion was demonstrated more exactly, of course, by the large deciduomata in the short-term experiments (Everett, 1954), and there seems no reason to expect any qualitative change in the corpus luteum product with advancing time.

Of the other trophic hormones, continuing production of only somatotrophin can be claimed with any certainty. Since the early reports by May (1935) and Martins (1936), maintenance or actual gain of body weight has been frequently noted heretofore in hypophysectomized rats bearing hypophyseal grafts. The marked atrophy of adrenal cortex and thyroids speaks for deficiency of ACTH and TSH, respectively. In the ovaries, atrophy of the follicular apparatus and interstitial tissue points to the near or complete absence of FSH and LH. In the short-term series (1954) such deficiencies were observed at even 8 days, and no difference could be seen between engrafted rats and hypophysectomized controls, in these respects.

Extreme gonadotrophin deficiency has been a common experience after pituitary transplantation or after the comparable procedure of stalk section without subsequent regeneration of hypophyseal portal veins (e.g. Westman and Jacobsohn, 1937, 1940; Harris, 1950; Harris and Jacobsohn, 1952; Cheng *et al.*, 1949; Fortier, 1951). There are certain important exceptions, however. Of especial interest, in the light of present observations, is the finding by Schweizer *et al.* (1937) that pituitary grafts in female guinea pigs, in the proven absence of glandular tissue in the sella turcica regularly produced a state of constant estrus associated with persistent ovarian follicles. An analogous effect seems to have occurred in some of the stalk-sectioned rats described by Greep and Barrnett (1951) in which estrogenic effects were noted, in company with antral ovarian follicles. Possibly the functional state of the gland at the time of transplantation or of stalk section determines the end result. Certainly the present findings do not deny the possibility of some moderate FSH and LH secretion after transplantation carried out under other circumstances. They merely demonstrate that the present circumstances favor luteotrophin production and prevent that of the other gonadotrophins.

The amount of luteotrophin secreted is perhaps best expressed by the sizes of the corpora lutea and constituent cells in animals of Group A, which received no estrogen treatment. We have seen that these corpora are essentially identical with those of normal pseudopregnancy or early pregnancy. Furthermore, the enlargement by estrogen administration compares with that which would be expected after such treatment of a normal pseu-

dopregnant rat. It would appear that production of luteotrophin in the engrafted rat is significantly greater than the small amount put out by the normal gland during the short cycle (Everett, 1945).

This increased output results simply from circumstances attendant on transplantation, since no special stimulus was administered. Transient irritation of the gland cells could hardly cause secretion to continue for the long periods observed. Nor is there reason to believe that the effect depends on any substance locally produced in the renal capsule. In a few instances the transplanted tissue accidentally slipped out and became functionally established a considerable distance away. Similarly, it may be recalled that in the short-term study two successful transplants were made to cervical fascia. Transplantation seemingly relieves the luteotrophin-secreting cells from some inhibitory influence to which they are subjected when in their normal location.

The persistence of active corpora lutea for several months brings us face to face with the possibility that, given appropriate stimulation, luteal cells may be capable of indefinitely continuous function, limited only by the aging or death of the individual. Certain it is in this one species that corpora lutea can function for at least 4 or 5 times the duration of pregnancy without evidence of progressive change. The contrary view has been repeatedly advanced that under any given extraovarian influence some inherent feature limits the time during which the luteal cells can respond (Astwood, 1941; Hisaw, 1944; Bradbury *et al.*, 1950).

The deterioration that normally occurs in corpora lutea of rats at the end of pseudopregnancy and pregnancy must depend on extraovarian mechanisms. The *arrest* of luteolysis in corpora lutea of the next youngest set (CL_{II}) in the experiments described here implies that the same is true with respect to corpora lutea of cycling rats.

The engrafted rat is well suited as a test animal in the search for luteolytic mechanisms. A small beginning has already been made. Obviously, removal of the source of luteotrophin produces prompt luteal atrophy. From the two animals of Group C that received repeated massive treatments with estrogen it is evident that such treatment is not luteolytic and that the enlarged luteal cells remain fully supported by the graft. It might otherwise have been thought that after hypertrophy the available luteotrophin would be inadequate by itself, once the estrogen pool had been exhausted.

As a working hypothesis one may suppose that in the normal cycling rat the hypothalamus, by way of its neurovascular linkage to the pars distalis, has a partial inhibitory effect on luteotrophin secretion. The inhibiting agent(s) may be the same that foster(s) secretion of other gonadotrophin(s). To enlarge on this with an attempt at detailed explanation of the luteal phase of the cycle and the more complex phenomena in the pregnant animal would be grossly premature.

SUMMARY

In 24 cycling adult female rats the pars distalis was transplanted into the renal capsule on the day after ovulation. The test for corpus luteum function and, indirectly, for luteotrophin secretion was vaginal mucification following administration of an excess of estrogen (125 μg. estradiol benzoate). This test was limited in typical experiments to the final week, whereas the total duration ranged from 23 to 120 days.

In each of 6 engrafted rats that remained untreated for 26 to 90 days a full set of corpora lutea was maintained at a diameter of ca. 1.5 mm. and the vaginal epithelium was atrophic. In 13 rats that were tested with estrogen during the final week, in experiments that extended for 23 to 104 days, the corpora lutea were enlarged to ca. 2.0 mm. and the vaginal epithelium was mucified. In 5 other rats that responded with vaginal mucification to estrogen tests during the third or fourth months, the graft was shortly removed and more estrogen was injected. This now caused vaginal cornification, and within 8 days the corpora lutea diminished to <1.2 mm. diameter with pronounced histologic evidence of degeneration. In all cases the ovarian follicular apparatus and interstitial tissue were atrophic.

Thus, removal of the hypophysis from its normal site favors luteotrophin secretion, and by eliminating luteolytic mechanisms allows corpora lutea to remain functional at least 4 or 5 times the duration of pregnancy. The functional life of rat corpora lutea is evidently not self-limiting, as often postulated.

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A COMPARISON OF THE EFFECTS OF GROWTH HORMONE AND OF INSULIN ADMINISTRATION

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AS A result of studies on protein metabolism in pregnant rats (Beaton *et al.*, 1954), we became interested in possible hormonal factors which might bring about the metabolic alterations seen after the fifteenth day of gestation. Another paper (Beaton *et al.*, 1955) has described the similarity between the changes seen in pregnancy and those produced by the administration of growth hormone to nonpregnant rats. It is well known that insulin administration results in an increased food intake and consequent body weight gain. It has been proposed (Salter and Best, 1953; Lawrence, Salter and Best, 1954) that insulin may have a growth hormone-like activity or that the two hormones may be closely interrelated. If this concept about insulin is correct, insulin might be the hormone responsible for the metabolic alterations evident in the pregnant rat. It was decided to test the effects of insulin administration in nonpregnant rats to see if a metabolic picture similar to that seen in pregnancy or after growth hormone treatment could be produced.

MATERIALS AND METHODS

Seventy female Wistar strain rats from the Carworth Farms colony were maintained on a 20% corn oil, 20% casein diet; this diet was prepared as described previously (Beaton *et al.*, 1953) except that a non-nutritive cellulose bulk was used in place of agar. Following a 10-day equilibration period, the rats were divided into 7 groups of 10 rats each and having an initial average body weight of 230 gm. per rat. The animals of two groups received single daily injections of 4 units protamine zinc insulin[1] per rat; those of two other groups received injections of 4 mg. growth hormone[2] per rat; those of two other groups received 0.25 ml. of 0.9% saline per rat. All injections were given subcutaneously. The seventh group was fasted and killed, without injection, as an initial control group. The experiment was carried on until 15 daily injections had been given to each rat; during this period all of the animals were provided with food and water *ad libitum*. On the day of the last injection, the dosages of growth hormone and insulin were reduced to one-half, and one hour after their administration, the rats of one of each pair of groups were killed. It should be noted that these animals were unfasted. The remaining rats were fasted for 18 hours and then killed approximately 24 hours after the last injection. This procedure was followed so that the effects of insulin could be studied during its administration; if this hormone was given to fasted rats, it is prob-

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[1] Protamine Zinc Insulin: Connaught Laboratories, 40 units per cc.

[2] Anterior Pituitary Growth Hormone: Armour, 8 mg. per cc.

able that severe hypoglycemia and death would follow. All animals were killed by stunning and decapitation and blood was collected from the neck in heparinized tubes.

Blood and enzyme analyses were carried out by the following procedures:—blood sugar, Nelson (1944); blood urea, Archibald (1945); blood amino nitrogen, Frame *et al.* (1943); packed cell volume by the standard procedure; aspartic-glutamic transaminase activity by the method of Tonhazy *et al.* (1950) and alanine-glutamic transaminase activity by this method as modified by Caldwell and McHenry (1953). After killing the fasted rats, the carcasses were pooled by groups, frozen and passed through a power grinder. The minces were mixed and samples were taken for determination of carcass constituents by the following procedures:—total crude fatty acids, Gavin and McHenry (1940); nitrogen by the standard macro-Kjeldahl procedure, multiplying by 6.25 to give the protein; moisture by the standard procedure of drying in aluminum dishes (A.O.A.C.) at 105°C. for 5 hours.

Statistical analyses were carried out by means of the "t"-test.

RESULTS AND DISCUSSION

During the experimental period, the insulin and growth hormone-treated groups gained weight at almost identical rates. At the end of the experiment, the weight gains were as follows: growth hormone-treated, 47 ± 9 gm. per rat; insulin-treated, 44 ± 9 gm. per rat; saline-treated, 11 ± 6 gm. per rat. These differences between the treated groups and the controls were significant at the 1% level. It should be noted that the average food intakes throughout the course of the experiment in gm. per rat per day were as follows:—growth hormone-treated, 11.6 gm.; insulin-treated, 15.7 gm.; saline-treated, 10.8 gm.

The results of blood and enzyme analyses are shown in Table 1. Among the non-fasted animals, insulin administration caused a marked hypoglycemia as might be expected, and a slight, but not significant lowering of blood urea and amino nitrogen. Growth hormone administration did not

TABLE 1. BIOCHEMICAL OBSERVATIONS FOLLOWING THE ADMINISTRATION OF GROWTH HORMONE OR OF INSULIN
Mean ±S.D.

Group	Liver weight (% body wt.)	Transaminase activity[1]		Blood assays		
		Alanine-glutamic	Aspartic-glutamic	Sugar (mg.%)	Urea (mg.%)	Amino nitrogen (mg.%)
Initial (fasted)	2.73 ±0.14	59 ±14	126 ±12	71 ±12	33.4 ±9.8	9.2 ±0.8
Final (fasted)						
Control	2.54 ±0.21	60 ±13	103 ± 7	52 ± 7	28.8 ±8.8	11.0 ±0.8
Growth Hormone	2.95 ±0.27	44 ±10	81 ± 5	47 ±12	26.4 ±7.8	14.7 ±1.1
Insulin	2.57 ±0.20	65 ±16	112 ±15	67 ± 5	32.3 ±9.2	10.7 ±1.1
Final (unfasted)						
Control	3.54 ±0.18	52 ±14	87 ±10	72 ± 9	33.8 ±7.0	10.6 ±0.9
Growth Hormone	4.15 ±0.40	33 ± 9	65 ± 6	72 ± 5	24.3 ±3.9	11.5 ±0.1
Insulin	3.07 ±0.22	66 ±14	105 ± 8	30 ± 9	28.3 ±4.4	9.2 ±1.1

[1] Transaminase activities are expressed as the Q(10/T), defined as the microliters of pyruvate—CO_2 formed per milligram wet tissue per hour.

affect the blood sugar, but lowered the urea and elevated the amino nitrogen; both of these changes were significant at the 1% level. The administration of growth hormone caused significant decreases in the activities of both of the liver transaminases; insulin administration had little effect on the alanine-glutamic transaminase, but significantly increased the activity of the aspartic-glutamic enzyme.

Among the fasted rats, a similar set of results was seen with respect to the growth hormone treatment. However, insulin administration no longer affected either of the transaminases, blood urea or blood amino nitrogen; there was a significant hyperglycemia among these rats, probably reflecting a transient diabetic state. In both fasted and unfasted rats, the liver weight was significantly increased by growth hormone administration; no effect

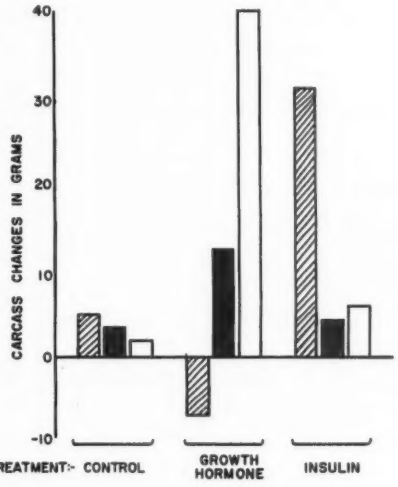

FIG. 1. Net Changes in Carcass Composition. The shaded areas represent fat, the solid areas represent protein and the open areas represent moisture. The values are calculated for the fasted rats and indicate the increase or decrease of the particular carcass constituent during the 14 days of injections.

was seen with insulin administration in fasted rats, but the weight was significantly lower in the unfasted insulin-treated rats than in the controls.

Biochemically, then, the effects of growth hormone and of insulin administration are quite different.

The results of carcass analyses afford an even greater contrast between the effects of the two hormones. The net changes in carcass composition during the injection period are shown in Figure 1. Most of the body weight increase of the insulin-treated rats can be accounted for by increased fat deposition with only a small increase in protein storage; growth hormone-treated rats show a net loss of fat stores and an increase in the carcass protein and moisture in approximately the ratio in which they appear in normal tissue. These findings might be anticipated from the records of food intake. During the 14 days of the experiment, the growth hormone-treated rats showed an average body weight gain of 35 gm. more than did the con-

trols, but ate only a total of 12 gm. more food. On the other hand, the insulin-treated rats showed an average body weight gain of 33 gm. more than did the controls, but ate a total of 69 gm. more food. Apparently then, the growth hormone-treated animals showed an economy of food utilization, whereas the insulin-treated rats showed merely a storage, as fat, of an excess caloric intake.

From the data obtained in this experiment it seems apparent that growth hormone and insulin administration produce quite distinct biochemical alterations in the intact female rat. Furthermore, although both of these hormones in the dosages given produced almost equal body weight increases, the composition of these gains was quite different. The data on carcass composition suggest that growth hormone administration stimulates fat catabolism so as to provide energy and thus spare protein. This concept is in agreement with the findings of Greenbaum *et al.* (1953). Certainly, the present experiment does not support the concept that growth hormone and insulin have a similar growth-stimulating effect. The data also indicate that insulin is not the hormone responsible for the metabolic alterations of pregnancy in the rat.

SUMMARY

The effects of growth hormone and of insulin administration on two liver transaminases, blood urea, amino nitrogen and sugar, and on carcass composition have been examined in intact female rats.

The data presented illustrate that the biochemical effects of these two hormones are quite distinct.

Although both treatments produced almost identical body weight increases, the gain in insulin-treated animals was nearly accounted for by fat deposition whereas the growth hormone-treated rats retained protein and moisture and showed a net loss of fat.

The changes in carcass composition suggest that growth hormone-treated rats exhibit an increased catabolism of fat to provide energy and spare protein. Insulin-treated rats simply store an excess caloric intake as body fat.

Acknowledgments

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AMPHENONE INHIBITION OF ADRENAL CORTICOSTEROID OUTPUT IN THE HYPOPHYSECTOMIZED DOG

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HERTZ *et al.* (1951) reported evidence of decreased corticosteroid production by the hypertrophied adrenals of the Amphenone[2]-treated rat. Hume and Nelson (1955a) demonstrated a marked depression in the rate of adrenal corticosteroid production in the acutely hypophysectomized dog following intravenous administration of Amphenone. These investigators used adrenal corticosteroid output in response to standardized doses of ACTH as a measure of adrenal cortical functional activity.

Studies made during the post-Amphenone treatment period in the hypophysectomized and the intact dog are presented in this report. Data are included which show the lack of effect of stilbestrol on the rate of corticosteroid output of the adrenal in the acutely hypophysectomized dog. In addition, the findings of Hume and Nelson (1955a) were confirmed.

MATERIALS AND METHODS

Eleven male and female mongrel dogs, weighing between 9 and 31 kg., were used in this study. Anesthesia was induced with intravenous nembutal solution using 27 mg. nembutal per kg. body weight. Hypophysectomy was performed by the transbuccal approach (McLean, 1928). Following hypophysectomy normal body temperature was maintained by the use of electric heating pads. A polyethylene catheter placed in the left femoral artery was connected to a resistance-type pressure transducer[3] which in turn was connected to an amplifying and recording system[4] for continuous blood pressure measurement. A polyethylene catheter placed in the right cephalic vein was used for the administration either of test compounds or of whole blood.

The procedure for cannulation of the right adrenal vein and for the collection of adrenal venous blood was that described by Hume and Nelson (1955b).

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[1] The material in this investigation will appear in a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology, Graduate Council, George Washington University.

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[3] Statham Pressure Transducer (Model P-23A).

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pletion of the Amphenone infusion a 10 cc. saline solution of ACTH (10 milliunits[5] in all but two cases) was given by the same route. The ACTH was followed by 10 cc. saline to flush any remaining ACTH from the cannula into the circulation. Three minutes later, adrenal blood samples were collected in heparinized 50 ml. graduated test tubes and stored overnight at 4.5° C. Blood taken during the procedure was replaced by infusion of citrated whole blood. Blood from the donor dog was collected from 1 to 5 days prior to use. The same procedure was used throughout in the experiments in which diethylstilbestrol dipotassium sulfonate was administered in aqueous solution.

Using whole blood, 17 hydroxycorticosteroids were determined by the method of Silber and Porter (1954) with modifications suggested by Peterson *et al.* (1955). These methods were slightly modified in order to adapt the procedure for handling whole blood in volumes ranging from 10 to 40 cc. Whole blood was used in order to obviate calculations of blood content from plasma determinations.

RESULTS

Series A

Adrenal Cortical Response to ACTH in the Acutely Hypophysectomized Dog Before and After Treatment with Amphenone. In a group of seven acutely hypophysectomized dogs in which each animal served as its own control, Amphenone was first tested against a 10 milliunit dose of ACTH. It may be seen by reference to the control samples in the figures that the corticosteroid increase resulting from a test dose of 10 milliunits of ACTH has usually disappeared within thirty minutes. The output of 17 hydroxycorticosteroid per 10-minute sample was significantly depressed following administration of Amphenone at levels of 25 and 50 mg. per kg. body weight. In the 10-minute interval after treatment with Amphenone, the response to a 10 milliunit dose of ACTH was reduced to about 20–25% of the response produced by the same dose of ACTH alone. This is in accord with the results reported by Hume and Nelson (1955a).

Figure 1 illustrates the response to ACTH at time intervals up to 3 hours after treatment with Amphenone at 50 mg. per kg. body weight. In each of the three animals the response to a standard dose (10 milliunits) of ACTH was still depressed 1 hour after treatment. Dog No. 255 shows corticosteroid suppression even at the end of 3 hours following Amphenone. In Dog No. 230 (Fig. 1) which received 25 mg. Amphenone per kg. body weight, a dose of 100 milliunits of ACTH (10 times the standard dose) given 1 hour after the initial inhibition was not capable of reversing the corticosteroid depressant action of Amphenone.

The time-response relationship is quite different in the intact dog in contrast to the hypophysectomized animal. Figure 2 indicates that even though a high dose of Amphenone (62.5 mg. per kg.) can markedly depress the endogenous corticosteroid output in the intact dog, the recovery from the depressant effect is fairly rapid. After 1 hour there is a seven-fold in-

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[5] One milliunit is 1/1000 U.S.P. Unit ACTH used was Wilson's Corticotropin Solution Lot #94091.

AMPHENONE INHIBITION OF ADRENAL CORTICOSTEROID OUTPUT IN THE HYPOPHYSECTOMIZED DOG

WILLIAM W. TULLNER,[1] MORRIS M. GRAFF AND ROY HERTZ

U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland

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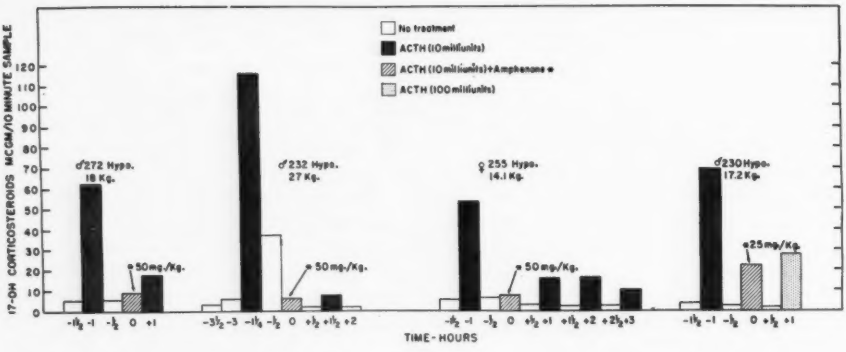

FIG. 1. Corticosteroid response to ACTH in the hypophysectomized dog before and after Amphenone treatment.

crease in rate of hormone production over that of the immediate post-Amphenone level and after 2 hours the output is essentially normal. In two instances of incomplete hypophysectomy a similarly rapid recovery of adrenal cortical function after Amphenone treatment has been noted.

Results obtained in an acutely hypophysectomized dog, in which a massive dose of ACTH was tested against the action of Amphenone, are shown in Figure 3. These results demonstrate that although Amphenone can

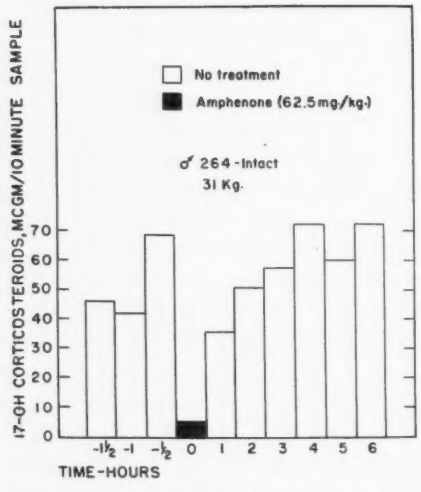

FIG. 2. Corticosteroid output in the intact dog before and after Amphenone.

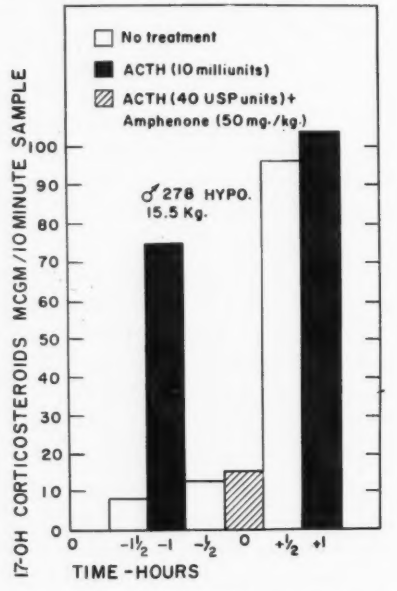

FIG. 3. Effect of ACTH level on adrenal corticosteroid output before and after Amphenone treatment.

exert its immediate blocking effect at a dose of ACTH 4000 times greater than the standard, thirty minutes later the corticosteroid level had exceeded the level produced by the pre-Amphenone test dose of ACTH. This large dose of ACTH reversed the delayed depressant effect noted earlier (Fig. 1) in which the same level of Amphenone but a very much smaller dose of ACTH (10 milliunits) was used.

Series B

Adrenal Corticosteroid Response to ACTH in the Acutely Hypophysectomized Dog After Treatment with Diethylstilbestrol.—Water soluble diethylstilbestrol dipotassium sulfonate was used as a control compound because of its structural relationship to Amphenone. Results on Dog No. 202 (Fig.

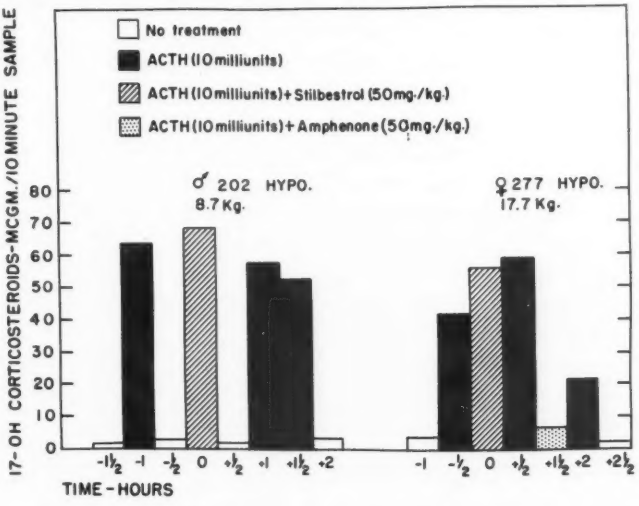

FIG. 4. Corticosteroid output in response to ACTH before and after stilbestrol treatment.

4) illustrate the lack of effect of diethylstilbestrol dipotassium sulfonate (50 mg. per kg. body weight) on corticosteroid output when the adrenal was challenged with a standard dose of ACTH. There was no evidence of delayed effects of diethylstilbestrol dipotassium sulfonate. In another experiment (Fig. 4, Dog No. 277), it is apparent that diethylstilbestrol dipotassium sulfonate will not reduce corticosteroid output in response to ACTH stimulation of the adrenal whereas in the same experimental animal, Amphenone is shown to have its marked depressant effect.

DISCUSSION

The ability of Amphenone to suppress 17-hydroxycorticosteroid production by the adrenal of the acutely hypophysectomized dog after ACTH

stimulation is in agreement with the findings of Hume and Nelson (1955a). Their studies established the suppression of corticosteroid production in the interval just after Amphenone administration.

Another aspect of the problem concerned the duration of corticosteroid blocking action of Amphenone. It is apparent from the experiments reported in Series A that the depressant effect persists for several hours in the acutely hypophysectomized dog. The dose of ACTH used (10 milliunits) was of such magnitude that its effects were usually dissipated within thirty minutes. Higher levels of ACTH have been shown to extend their effects over increasing periods of time (Nelson and Hume, 1955).

When one examines the results on the intact dog, it is evident that where there is a continuous endogenous source of ACTH the initially reduced corticosteroid output evoked by Amphenone returns to normal levels within one or two hours.

In the case of an acutely hypophysectomized dog in which a large (40 U.S.P. units) dose of ACTH is given just after Amphenone, the initially depressed rate of corticosteroid production occurs but as in the intact animal, the inhibition is shortly reversed. With the increased duration of action produced by this level of ACTH, corticosteroid production is at a peak both at thirty minutes and at one hour following Amphenone treatment. Thus it is clear that a high circulating level of ACTH whether of exogenous or endogenous origin can overcome the persistent blocking action of Amphenone seen in the acutely hypophysectomized dog when lower doses of ACTH are given.

The experiments with diethylstilbestrol point up the specificity of Amphenone in its depressant effects on adrenal cortical hormone production but leave the mode of action on the metabolic pattern unexplained.

SUMMARY

The rate of 17 hydroxycorticosteroid output in the adrenal vein of the hypophysectomized dog in response to a 10 milliunit dose of ACTH was markedly depressed following treatment with Amphenone. Stilbestrol dipotassium sulfonate used for comparison with Amphenone showed no corticosteroid inhibitory action.

This depressant effect of Amphenone on corticosteroid production persisted as much as three hours after treatment.

In the intact dog after the initial corticosteroid depressant action of Amphenone, there is a fairly rapid return of corticosteroid output to a pretreatment level. A similar pattern is seen in dogs receiving massive doses of ACTH.

Acknowledgment

The authors wish to acknowledge the invaluable laboratory assistance of Mr. Donald Barber and Mr. Silas Jackson.

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INFLUENCE OF OXYCEL CORTICOTROPIN ON FATTY ACID AND GLUCOSE METABOLISM IN THE RAT[1]

MILDRED G. ENGEL AND FRANK L. ENGEL

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REPORTS have emanated from two laboratories showing that certain preparations of corticotropin have striking metabolic effects in both the rat and mouse. These include mobilization of depot fat to the liver, ketonemia, an increase in oxygen consumption, a depression of the R. Q., insulin resistance and a fall in the blood sugar (Astwood, 1955a,b, Engel, 1955; Engel and Engel, 1954; Rosenberg, 1953; Westermeyer and Raben, 1954). Certain of these responses have been demonstrable in adrenalectomized animals, indicating that they are not mediated by the adrenal cortex. In the past, they have been attributed to the pituitary fraction labeled growth hormone, but in our experience, as well as that of Astwood's group, oxycel corticotropin has exhibited much greater stability and activity with respect to these metabolic properties than have those growth hormone preparations which have been available to us. Thus, we have regularly found a ketogenic response to oxycel corticotropin in a dose of 30 μg. or less in the anesthetized rat whereas the minimal dose of the most active growth hormone sample tested for ketogenic activity was 100 μg. Doses of from 300 to 3000 μg. of even highly purified growth hormone have been required to produce ketonemia comparable to that from corticotropin. Twenty-five to fifty micrograms of certain of these same growth hormone samples readily stimulated widening of the tibial epiphysis in the hypophysectomized rat (Engel, 1955). The precise nature and identity of the metabolic factor associated with corticotropin remains to be established.

The mechanism by which the pituitary factor associated with corticotropin induces ketosis and lowers the blood sugar is as yet poorly understood. The purpose of the present report is to describe some additional findings concerning the metabolic activity of this material. Oxycel corticotropin was found to increase ketonemia during an infusion of sodium octanoate, to prevent the antiketogenic effect of glucose and to increase tolerance to glucose in the intravenous glucose tolerance test in the fasted rat.

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METHODS

The source and sex of the rats, diet, handling and the analytical methods were the same as in previous reports (Engel and Engel, 1954; Scott and Engel, 1953). Blood ketone bodies were determined as described elsewhere except that the modification of Michaels was not used (Werk, *et al.* 1955).

After an overnight fast, the rats, which weighed between 200 and 300 gm. were anesthetized with nembutal and placed in a constant temperature box at 33°C. for 30 minutes. At the end of this time, the external saphenous vein was connected to an infusion pump. During the next 30 minutes different groups of rats received intravenously per 100 gm. body weight 1 ml. of the following solutions: 0.9% saline, 1.5% sodium octanoate, 10% glucose, or 1.5% sodium octanoate in 10% glucose. During the infusion period the animals were kept at room temperature while during the 30 minutes before and after infusion they were replaced in the constant temperature box.

The corticotropin treated rats were given Wilson's oxycel corticotropin (lot 90834) intraperitoneally in a dose of 0.120 mg. 30 minutes before infusion. Tail blood was drawn from all animals at 0, 30, 60, and 90 minutes and analyzed for ketone bodies and glucose.

RESULTS

Table 1 shows the blood ketone levels and Table 2 the blood sugar levels in the 4 corticotropin treated groups and their corresponding controls. The increments and decrements in the blood ketone levels during the various experimental periods are recorded in the last 3 columns of Table 1.

During the 30 minutes before the intravenous infusions all the corticotropin treated groups showed significant increases in the blood ketone levels whereas the controls showed no significant change. This rapid ketonemic response to corticotropin confirms our previous report (Engel and Engel, 1954). All groups showed a significant increase in blood glucose levels during this same interval irrespective of corticotropin treatment. This is presumably an epinephrine effect related to handling and induction of anesthesia, since it does not occur in the adreno-demedullated rat (Unpublished observations).

TABLE 1. EFFECT OF OXYCEL CORTICOTROPHIN ON BLOOD KETONE LEVELS

Experimental groups	No. of rats	Blood ketone levels as mg. percent acetone (Mean±SE)						
		Infusion				Infusion		
		0	30 min.	60 min.	90 min.	0-30 min.	30-60 min.	0-90 min.
1. Saline Control	12	7.07±0.45	7.40±0.49	8.39±0.58	8.67±0.58	+0.32±0.35	+1.00±0.26*	+1.63±0.37*
2. Octanoate	10	5.25±0.38	5.31±0.38	7.66±0.46	7.62±0.28	+0.06±0.30	+2.35±0.42*	+2.37±0.34*
3. ACTH-Saline	14	5.00±0.44	6.84±0.60	9.90±0.70	11.44±0.81	*+1.84±0.31**	*+3.06±0.35**	*+6.44±0.56**
4. ACTH-Octanoate	12	5.64±0.23	7.22±0.44	14.16±0.67	14.96±0.62	*+1.58±0.35**	*+6.94±0.57**	*+9.39±0.64**
5. Glucose Control	12	6.65±0.64	6.64±0.87	1.16±0.44	3.37±0.54	−0.01±0.31	−5.49±0.75*	−3.28±0.63*
6. Octanoate-Glucose	11	6.34±0.55	6.57±0.71	2.37±0.20	3.06±0.64	+0.23±0.30	−4.20±0.57*	−3.28±0.53*
7. ACTH-Glucose	15	6.09±0.41	8.00±0.42	7.14±0.81	9.06±0.75	*+1.91±0.23**	*−0.86±0.80*	*+2.97±0.77**
8. ACTH-Octanoate-Glucose	14	5.78±0.50	7.83±0.59	7.63±1.18	8.46±0.84	*+2.05±0.37**	*−0.20±0.90*	*+2.68±0.80**

* $P<.01$.

Italicized values are significant ($p.<01$) compared to corresponding controls not receiving ACTH. Only the changes in blood ketones are included in the statistical analysis.

During the 30 minute infusion of saline there was a small but statistically significant increase in the blood ketone levels (1.00 ± 0.26 mg.%) and in the following 30 minutes a further small rise. The total change in blood ketone level in 90 minutes was $+1.63 \pm 0.37$ mg.%. In the animals that received the octanoate, the increment during the infusion amounted to 2.35 ± 0.42 mg.%, the difference between this and the control presumably representing conversion of the fatty acid to ketone bodies. In the period after infusion there was no further rise in blood ketone values confirming earlier observations with sodium octanoate infusion in the rat (Engel and Hewson,

TABLE 2. EFFECT OF OXYCEL CORTICOTROPIN ON BLOOD GLUCOSE LEVELS

Experimental group	No. of rats	Mean ±SE			
		Blood glucose in mg. per cent			
			Infusion		
		0	30 min.	60 min.	90 min.
1. Saline Group	6	73 ±2.0	91 ±4.0*	88 ± 4.8*	83 ±3.6
2. Octanoate	5	71 ±2.7	95 ±4.3	83 ± 2.3*	85 ±5.1
3. ACTH-Saline	8	77 ±5.1	100 ±4.5*	79 ± 3.9	78 ±3.8
4. ACTH-Octanoate	6	80 ±1.2	106 ±4.8*	68 ± 2.7*	*68 ±2.7**
5. Glucose Control	12	77 ±1.8	90 ±2.9*	216 ± 5.1*	119 ±5.8*
6. Octanoate-Glucose	11	80 ±3.5	95 ±2.9*	222 ±11.3*	118 ±7.3*
7. ACTH-Glucose	15	75 ±1.4	91 ±2.6*	*175 ±10.0**	*84 ±5.8*
8. ACTH-Octanoate-Glucose	14	76 ±2.2	97 ±2.6*	*168 ± 9.1*	*90 ±6.6*

* Significant ($p < .01$) compared to initial blood sugar.
Italicized values are significant ($p < .01$) compared to corresponding controls not receiving ACTH.

1953). The corticotropin-treated group showed a rise of 3.06 ± 0.35 mg.% during the saline infusion and a net rise of 6.44 ± 0.56 mg.% during the entire experiment. The rats which were pretreated with corticotropin and received an intravenous infusion of sodium octanoate exhibited the greatest increase in ketones both during the infusion (6.94 ± 0.57 mg.%) and during the whole experiment (9.31 ± 0.64 mg.%).

During glucose infusion, with or without octanoate, there was a sharp decline in blood ketone levels. This fall was a little less in the octanoate group than in the glucose group, but the difference was not statistically significant. The antiketogenic effect of glucose did not extend beyond the glucose infusion period. In contrast to the glucose controls, the corticotropin treated animals showed no decline in ketonemia during the glucose or glucose-octanoate infusion, and both showed a net increase in ketone levels at 90 minutes. Thus, corticotropin either prevented the fall in blood ketone levels ordinarily observed during glucose infusion or conversely glucose overcame the usual ketonemic response to corticotropin and hence, there was no net change in blood ketone levels.

Figure 1 summarizes the changes in blood ketones during the 30 minute infusion period in all groups. The bar graphs are plotted upward for the animals not receiving glucose and downward for the groups infused with

glucose. The line drawn even with the rise in the saline control group (+1.00 mg.%) is taken as the base line for all changes since with no treatment this degree of ketonemia would be anticipated in all groups.

It is apparent from this graph that the increase in blood ketones in the group which received corticotropin and octanoate is significantly greater than all other groups and exceeds that which would be expected if the effects of hormone and octanoate were additive (column 5).

The bars plotted downward illustrate the antiketogenic effect of glucose. In the presence of glucose there was no clearly demonstrable ketone response from octanoate in any series. The glucose effect may be visualized by considering the total length of the bars as indicative of the antiketogenic effect. In these terms, all but column 3 (corticotropin) had approximately equal falls from the level of ketones which would have been reached had

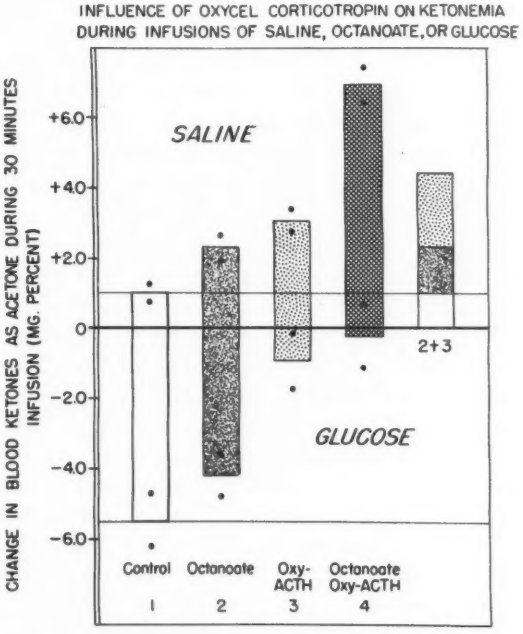

FIG. 1. The effects of oxycel corticotropin on ketonemia in rats infused intravenously with saline and/or sodium octanoate are plotted above the base line while those receiving glucose and sodium octanoate are plotted below the base line. Groups 1 and 3 received either saline or glucose intravenously while Groups 2 and 4 received either sodium octanoate or 10 per cent glucose in sodium octanoate. The portions of the increments plotted from the top of the saline control represent the response to the treatments. The distances from the line drawn from the bottom of the glucose control to the bottoms of the columns from the other groups may be interpreted as a measure of the effects of octanoate and/or corticotropin on ketonemia in the presence of glucose. The last column (5) indicates the change expected from sodium octanoate and corticotropin were they additive.

glucose not been given. The reason for the difference between the two corticotropin groups is not apparent. The corticotropin response in the presence of glucose can be visualized in another way, namely by plotting the distance from the lowest base line to the bottoms of columns 3 and 4 as being indicative of the relative ketogenic effect of the hormone in the presence of glucose. In these terms one can say that corticotropin had a relative ketogenic effect in the presence of glucose and this was not modified by the simultaneous infusion of octanoate.

The blood sugar levels in the four groups not receiving glucose were unremarkable except that the 60 and 90 minute values of the corticotropin-octanoate group were lower than the other groups (Table 2). In the glucose infusion groups, however, the blood sugar levels at the end of the infusion (60 minutes) as well as 30 minutes later were significantly lower in the corticotropin treated animals. In short, corticotropin improved the tolerance of the rat to intravenously administered glucose.

DISCUSSION

Elsewhere we have discussed the possible mechanism by which growth hormone induces ketosis (Engel, 1955). Although it is not established whether the ketogenic responses to corticotropin and growth hormone are due to the same hormonal principle, the same mechanisms may be considered for corticotropin as for growth hormone in this discussion. It should be emphasized again that the ketonemia and hypoglycemia have been shown to occur in adrenalectomized rats receiving corticotropin and hence the observed effects cannot be attributed to stimulation of the adrenal cortex. The ketonemia might be due to (1) increased mobilization of fat from the periphery to the liver with a consequent increase in the substrate for ketone body formation without any change in fatty acid metabolism or ketogenesis *per se*. (2) A direct effect on fatty acid catabolism simultaneous with or independent of increased mobilization of fat to the liver. (3) An inhibition of carbohydrate utilization or a stimulation of glycogen breakdown in the liver with secondary accentuation of ketone body formation. (4) An inhibition of gluconeogenesis from protein with secondary carbohydrate depletion in the liver. (5) An inhibition of ketone body utilization by extra hepatic tissues.

Both oxycel corticotropin and growth hormone do have a well documented effect on fat mobilization from the periphery to the liver. This is demonstrable within the time noted for ketonemia in these experiments (Rosenberg, 1953; Greenbaum and McLean, 1953) and hence mechanism (1) could be operative. However, the isolated observations of Shipley (1944) and Tepperman and Tepperman (1951) that some samples of growth hormone increase acetoacetate production when added directly to liver slices *in vitro* indicate that with growth hormone fat mobilization is not a prerequisite to ketone body formation by the liver. The same might be true for corticotropin but this remains to be demonstrated.

The observation that ketonemia is considerably enhanced during octanoate infusion in the corticotropin treated animal suggests an action of the hormone on fatty acid catabolism and/or conversion to ketone bodies. This is apparent from the fact that ketonemia in this group exceeds that anticipated from the additive effects of corticotropin stimulation and octanoate conversion to ketone bodies. It is difficult to account for these data solely on the basis of fat mobilization. Further studies with the aid of isotopically labeled fatty acids are indicated to determine whether there is in fact an increased conversion of octanoic acid to ketone bodies under these experimental conditions. An observation having bearing on this point is that of Maassen (1952) who showed that liver slices from growth hormone treated rats lost more fat on *in vitro* incubation than did those from untreated animals, suggesting accelerated metabolism of liver fat.

These experiments throw no light on the question of whether corticotropin might exert its ketogenic effect by causing carbohydrate depletion in the liver or by inhibition of carbohydrate utilization of this organ. However, unpublished observations from this laboratory have revealed that 3 hours following the injection of corticotropin, there is no change in the liver glycogen levels of fasted animals which develop ketosis and a fall in blood sugar (controls 0.098 ± 0.052, corticotropin 0.166 ± 0.408 gm.%). The finding that intravenously administered glucose does not lower blood ketone levels in the corticotropin-treated rats, and yet that the glucose is more rapidly disposed of is not explicable in terms of a primary defect in hepatic carbohydrate metabolism. The failure of corticotropin to lower liver glycogen values is in disagreement with the results of Lotspeich and Petersen (1954, 1955) who found ketonemia and increased ketone production by liver slices *in vitro* to be associated with liver glycogen depletion in growth hormone or TSH-treated rats. However, Illingworth and Russell (1951) and Russell and Bloom (1956) found no comparable effect of growth hormone on liver glycogen in their studies.

Russell (1955) has found that oxycel corticotropin decreases the conversion of amino acids to urea and hence diminishes gluconeogenesis. While this might be a factor in ketosis in the animals not receiving glucose, it should be of no importance in the glucose treated animals, and hence would seem a most unlikely explanation for the ketonemia.

None of these data rule out an effect of the hormone on the peripheral utilization of ketone bodies. Further studies are needed to establish whether such an effect might exist.

Both growth hormone (Milman and Russell, 1950, Lotspeich and Petersen, 1954) and corticotropin (Westermeyer and Raben, 1954, Engel and Engel, 1955) induce hypoglycemia in fasted normal, adrenalectomized and hypophysectomized mice and rats. Most investigators agree with Milman and Russell that growth hormone has little or no effect on the blood sugar of fed animals or of fasted animals presented with a glucose load. However, Maassen (1952) has reported an impressive increase in glucose tolerance

immediately after treating rats with growth hormone, a response entirely comparable to that described in this report on corticotropin. As have others before and since, Maassen attributes the hypoglycemia and improved glucose tolerance to a stimulation of insulin secretion by growth hormone. This interpretation is consistent with the observation that growth hormone induces hyperglycemia rather than hypoglycemia in alloxan diabetic rats (Milman and Russell, 1950). It is called into question, however, by the demonstration that hypoglycemia can still be induced by growth hormone immediately after surgical pancreatectomy in the dog (Kurtz, De Bodo, Kiang and Ancowitz, 1951). This last experiment does not rule out a permissive effect of insulin. There are other reasons, too, for being cautious about attributing the hypoglycemic effect of either growth hormone or corticotropin to insulin. Corticotropin immediately stimulates ketonemia and prevents the antiketogenic effect of glucose. In contrast, insulin has a prompt antiketogenic effect, although ketosis may develop several hours after the hypoglycemia (Amatruda and Engel, 1956). The hypoglycemic effect of corticotropin was found by Westermeyer and Raben to be still demonstrable in rats which were insensitive to large doses of exogenous insulin. Insulin stimulates glycogen accumulation in adipose tissue while growth hormone in a dose causing comparable hypoglycemia has no such effect and does not modify the insulin response (Scott and Engel, 1950).

In our present state of ignorance, however, it would be hazardous to state that insulin played no role in these metabolic responses to growth hormone and corticotropin. Regardless of the place of insulin in this picture it is tempting to relate the hypoglycemia and improved carbohydrate tolerance to the stimulating effect of growth hormone on muscle glycogen accumulation in the rat (Russell and Bloom, 1956) and on the uptake of glucose by the rat diaphragm *in vitro* (Park, *et al.*, 1952). It is obvious, however, that considerably more information is needed before the interpretation of our results can be any more than speculative.

Claims have now been made attributing the ketogenic activity of anterior pituitary extracts to growth hormone, thyroid stimulating hormone (Petersen and Lotspeich, 1955) and corticotropin. Our own data concerning this aspect of the problem will be the subject of another report. It might be noted that the significance of the debate concerning the identity of the pituitary ketogenic factor is clouded somewhat by the recent demonstration that hypophysectomized rats develop ketosis in response to insulin hypoglycemia and fasting quite as rapidly and almost as intensely as do intact rats (Amatruda and Engel, 1956).

SUMMARY

Oxycel corticotropin induces a significant ketonemia within 30 minutes in the fasted rat and increases ketonemia during an intravenous infusion of sodium octanoate to a greater degree than predicted from the additive ketonemic effects of fatty acid and corticotropin administered separately.

Intravenously infused glucose immediately lowers blood ketone levels in the normal fasted rat, but not in fasted rats which have received oxycel corticotropin 30 minutes previously. Glucose treatment delays but does not completely prevent the usual ketonemic response to corticotropin.

Oxycel corticotropin increases the tolerance of the fasted rat to intravenously infused glucose.

These observations are interpreted as being either extra-adrenal effects of corticotropin or responses to a contaminant associated with the oxycel corticotropin since it has been previously shown that ketonemia and hypoglycemia could be induced by this agent in the adrenalectomized rat.

The possible mechanisms by which the metabolic activities of oxycel corticotropin are mediated are discussed.

Acknowledgments

We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Illinois, for generous quantities of oxycel corticotropin and the Armour Laboratories, Chicago, Illinois, for the sterile 1.5% sodium octanoate solution.

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore; 1956, Dr. Frederick L. Hisaw.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross; 1956, Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1956.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.

Schering Scholar, 1956: Dr. C. N. H. Long, Professor of Biochemistry, Yale University School of Medicine.

Upjohn Scholar, 1956: Dr. S. B. Barker, Professor of Pharmacology, University of Alabama Medical Center.

Citation for the 1956 Award of The Medal of the Endocrine Society

The highest honor within the capacity of The Endocrine Society to bestow, its medal, goes this year to Dr. Frederick L. Hisaw in recognition of his many contributions to endocrinology.

Dr. Hisaw was born in Missouri and pursued his education there. He began his teaching of zoology at the University of Mississippi, moving on to Kansas State College, before going to the University of Wisconsin. Here he rose rapidly to the rank of professor. In 1935, Dr. Hisaw was appointed Professor of Zoology at Harvard University where he now holds in addition the Fisher Chair in Natural History. He is also Senior Fellow in the Society of Fellows. Dr. Hisaw is a member of the National Academy of Sciences and of the American Philosophical Society. Ample witness is here given to his breadth and his distinction in the field of science.

True to his heritage from a people who had moved boldly into new lands, Dr. Hisaw from the beginning has struck out on his own into unexplored fields of biology. His early studies established that the sexual dimorphism of the pelvic girdle of the pocket gopher and the guinea pig was under hormonal control. He discovered and named *relaxin*, a substance that induces, at term, mobility of the symphysis pubis of pregnant guinea pigs. In quick order, his pioneering studies led to deeper insight into the fundamental nature of the hormonal interrelationships between the pituitary body and the ovary in the regulation of cyclic reproductive functions. All of these original investigations helped to bring glandular physiology into the scientific sun and led the way to the experimental exploitation of this field, the ramifications and richness of which are still unfathomed.

Throughout this period, Dr. Hisaw continued to play a leading role by shifting his points of attack as new knowledge and improved methods gave him opportunity. Today he is working at the forefront in a new approach, as evidenced in his recent publication entitled, "The Action of Estrogens upon Lactic Acid Oxidizing Enzyme Systems of Rat Uterus." His work alone has done much to bring endocrinology to its present high estate.

The breadth of his intellectual powers and his insight into the problems of others have made Dr. Hisaw a gifted teacher. He is also an extraordinary exponent of endocrinology from a philosophical as well as a physiological point of view. The honor which the award of this medal to Dr. Hisaw denotes is richly deserved.

Citation for the 1956 Ciba Award

Dr. Alfred M. Bongiovanni is the recipient of the Ciba Award for 1956, presented by The Endocrine Society.

Dr. Bongiovanni inherited a dominant gene of scientific curiosity from a mother who held a Doctor's degree in pharmaceutical chemistry. While working for his B.S. degree at Villanova College, he spent two summers at the Marine Biological Laboratories at Woods Hole fragmenting eggs of the sea urchin and showed that the denucleated fragments could be fertilized and would develop to the eight-cell stage. As a medical student at the University of Pennsylvania, he worked at night in the laboratories of Dr. Edmund Farris of the Wistar Institute and produced neurogenic hypertension in rats by means of audiogenic stimulation.

During his medical course at Pennsylvania, from which he graduated in 1943, he came to appreciate through the inspiration of Doctors Mitchell Rubin and Joseph Stokes the desirability of applying new and exact laboratory techniques in advancing the knowledge of human disease, and because of them he decided to enter pediatrics. After a rotating internship, two and one-half years on a Navy destroyer, and a year in experimental pathology, he spent two years in pediatric residence at the Children's Hospital, Philadelphia. He then felt a need for more laboratory training and entered the Rockefeller Institute in 1949.

At the Rockefeller, Doctors Henry Kunkel, Herbert Jaffe and others on the staff of Doctor Reginald Archibald encouraged him to study the metabolism of steroids in patients with liver disease. Here he acquired the rigid and meticulous methodology necessary for the studies of steroids.

In 1952 he was invited by Doctor Lawson Wilkins to join the research group in pediatric endocrinology at Johns Hopkins, particularly to study the steroidal patterns and pathways of steroidogenesis in patients with virilizing hyperplasia of the adrenals. A summer in the laboratories of Doctor Seymour Liebermann prepared him further for this. It was necessary for him to apply and improve methods for the fractionation and identification of steroids in the urine and blood. For two years tedious and painstaking studies were made on normal subjects and patients with the adrenogenital syndrome, Cushing's syndrome and other adrenal disorders. Bongiovanni's demonstration that pregnanetriol is increased in virilizing adrenal hyperplasia and that the plasma corticosteroids are increased little or none when ACTH is administered, lent further credence to the theory that this syndrome is due to defects in the hydroxylations necessary to convert 17-hydroxyprogesterone to hydrocortisone. During the past year, since returning to the University of Pennsylvania, he has shown that patients with virilizing adrenal hyperplasia and hypertension have a different pattern of steroidogenesis consisting of a specific defect of C_{11} hydroxylation which results in the formation of large amounts of desoxycorticosterone and compound S. These studies, and those of other workers, have clarified to a great extent the pathogenesis of the adrenogenital syndrome.

THE EIGHTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

Sponsored jointly by the Endocrine Society; The University of Texas Postgraduate School of Medicine, and the University of Texas M. D. Anderson Hospital and Tumor Institute,
Houston, Texas.
October 2–27, 1956

The faculty will consist of twenty-two eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program and registration, write to Dr. J. B. Trunnel, M. D. Anderson Hospital, 6723 Bertner Avenue, Houston 25, Texas. Registration is limited to 100; tuition fee is $100.00. Rooms will be reserved for the students and faculty at the Shamrock Hilton Hotel which is adjacent to the Texas Medical Center.

NATIONAL INSTITUTES OF HEALTH

NOTICE

The Public Health Service has announced a new procedure to expedite the processing of research grant applications for those requests which do not exceed $2000 plus indirect costs and which do not ask support for more than one year. Such applications will be accepted and processed on receipt and are not therefore subject to the usual deadlines for submission prior to review.

Council recommendations can be expected on these applications within 1–4 months from the time of submission. These procedures do not apply for requests for supplements to existing grants.

Address all applications as well as requests for forms or additional information to the Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.

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The Dickinson Research Memorial, research arm of the Planned Parenthood Federation of America, Inc., is interested in supporting basic research in the physiology of reproduction with special emphasis on factors influencing fertility. For further information address the Dickinson Research Memorial, 501 Madison Avenue, New York 22, New York.

AUTHOR INDEX, VOLUME 58

ADAMSONS, KARLIS, JR., STANFORD L. ENGEL, H. B. VAN DYKE, BODIL SCHMIDT-NIELSEN, AND KNUT SCHMIDT-NIELSEN, 272

ALBERT, A., see MOLNAR, GEORGE D., 381

ALBERT, A., see MOLNAR, GEORGE D., 501

ALLARD, CLAUDE, see WEBER, GEORGE, 40

ANGELAKOS, E. T., see DEUTSCH, S., 33

ARONSON, LESTER R., see WARREN, ROSLYN PAUKER, 293

AUSTIN, JANE, see KOCHAKIAN, CHARLES D., 315

BAKKE, JOHN L. AND NANCY LAWRENCE. The Effect of Thyroid Stimulating Hormone upon the Iodide Collecting Mechanism of Thyroid Tissue Slices, 531

BANKS, ROBERT C., see FARRELL, GORDON L., 104

BAUM, GENEVA, J. AND ROLAND K. MEYER. Influence of Diethylstilbestrol on Lipids in Intact and Hypophysectomized Cockerels, 338

BAVETTA, L. A., S. BERNICK AND B. H. ERSHOFF. Selective Effects of Growth Hormone on Rats Fed a Tryptophan-Low Diet, 701

BEATON, GEORGE H. AND DORIS M. CURRY. A Comparison of the Effects of Growth Hormone and of Insulin Administration, 797

BENIRSCHKE, KURT, ERIC BLOCH AND ARTHUR T. HERTIG. Concerning the Function of the Fetal Zone of the Human Adrenal Gland, 598

BENIRSCHKE, K., see BLOCH, E., 626

BENNETT, LESLIE L., see HAZELWOOD, ROBERT L., 427

BERNICK, S., see BAVETTA, L. A., 701

BEVER, ARLEY T., JOSEPH T. VELARDO AND FREDERICK L. HISAW. Action of Estrogens on Lactic Dehydrogenase-DPNH Oxidase System of Rat Uterus, 512

BEYLER, A. L., E. D. NEUMANN AND D. F. BURNHAM. The Biological Activity of a New DCA Derivative $\Delta^{1,4}$-Pregnadiene-21-OL-3, 20-Dione, 3-Acetate, 471

BLOCH, ERIC, see BENIRSCHKE, KURT, 598

BLOCH, E., K. BENIRSCHKE AND E. ROSEMBERG. C_{19} Steroids, 17α-Hydroxycorticosterone and a Sodium Retaining Factor in Human Fetal Adrenal Glands, 626

BLOOM, WALTER, see RUSSELL, JANE A., 83

BOIS, PIERRE, see SELYE, HANS, 739

BOWMAN, ROGER H. The Influence of the Adrenal Medulla on Muscle Glycogen in Normal and Hypophysectomized Rats, 158

BRENEMAN, W. R. Steroid Hormones and the Development of the Reproductive System in the Pullet, 262

BROWMAN, LUDVIG, G., see GRIESER, Kenneth Charles, 206

BROWN, HERBERT E. and THOMAS F. DOUGHERTY. The Diurnal Variation of Blood Leucocytes in Normal and Adrenalectomized Mice, 365

BROWN, JOSIAH. Extra-thyroidal Iodide Metabolism in the Rat, 68

BURNHAM, D. F., see BEYLER, A. L., 471

BURNS, THOMAS, W. Endocrine Factors in the Water Metabolism of the Desert Mammal, *G. gerbillus*, 243

CANTERO, ANTONIO, see WEBER, GEORGE, 40

CARLO, J., see HAGOPIAN, M., 387

CHAIKOFF, I. L., see TAUROG, ALVIN, 121

CHAIKOFF, I. L. see TAUROG, ALVIN, G., 132

CHEEVER, ELIZABETH, see LEVEY, HAROLD A., 420

CHERNICK, SIDNEY S. AND JANET, G. MOE. Effects of Hypophysectomy on the Coenzyme A Content of Rat Liver, 280

CLIFTON, KELLY H. and ROLAND K. MEYER. Effect of Food Intake on the Secretion of Thyrotrophin during Diethylstilbestrol Treatment, 681

CLIFTON, KELLY H., see MEYER, ROLAND K., 686
CLUGSTON, HELEN, see RUGH, ROBERTS, 8
COALSON, ROBERT, see KOCHAKIAN, CHARLES D., 315
COLLINS, ELLIOTT J. Steroid-Induced Adrenal-Pituitary Hypofunction, 777
CONDON, GEORGE P., see LEVITZ, MORTIMER, 376
CURRY, DORIS M., see BEATON, GEORGE H., 797

DANCIS, JOSEPH, see LEVITZ, MORTIMER, 376
DE LAMIRANDE, GASTON, see WEBER, GEORGE, 40
DEUTSCH, S., E. T. ANGELAKOS AND E. R. LOEW. A Quantitative Method for Measuring Melanophore Expanding Activity, 33
DIAMOND, MARIAN CLEEVES. The Effect of Hypophysectomy, Removal of the Posterior Pituitary, Adrenalectomy, and Dehydration on the Antidiuretic Activity of the Rat Hypothalamic-neurohypophyseal System, 461
DOCKRELL, ALICE W., see MILLER, RICHARD A., 550
DONE, ALAN K., see GOOD, THOMAS A., 651
DORFMAN, RALPH I., see ROSENFELD, GEORGE, 255
DORFMAN, RALPH I., see ROSEMBERG, EUGENIA, 708
DORFMAN, RALPH I., see KORITZ, SEYMOUR B., 748
DOUGHERTY, EDITH, see KOCHAKIAN, CHARLES D., 315
DOUGHERTY, THOMAS F., see BROWN, HERBERT E., 365
DRAGER, GLENN A., see GREGORY, RAYMOND, L., 79
DRILL, VICTOR A., see SAUNDERS, FRANCIS J., 567
DURY, ABRAHAM, see DURY, MURIEL., 671
DURY, MURIEL AND ABRAHAM DURY. Effects of Ascorbic Acid Pretreatment on the Response of Blood Glucose and Adrenal Cholesterol in the Intact Rat to Insulin, 671

EGDAHL, RICHARD H., see NELSON, DON H., 309
ELIAS, HANS AND JOHN E. PAULY. The Structure of the Human Adrenal Cortex, 714
ELY, ROBERT S., see GOOD, THOMAS A. 651
ENDAHL, GERALD L., see KOCHAKIAN, CHARLES D., 226
ENGEL, FRANK L., see ENGEL, MILDRED G., 808
ENGEL, MILDRED G. AND FRANK L. ENGEL. Influence of Oxycel Corticotropin on Fatty Acid and Glucose Metabolism in the Rat, 808
ENGEL, STANFORD L., see ADAMSONS, KARLIS, JR., 272
ERSHOFF, B. H., see BAVETTA, L. A., 701
ERWIN, HOWARD L., see GREER, MONTE A., 665
EVANS, W. W., see KOCHAKIAN, CHARLES D., 279
EVERETT, JOHN W. Functional Corpora Lutea Maintained for Months by Autografts of Rat Hypophyses, 786

FARAH, A., F. KODA AND M. FRAZER. Studies on the Control of the Renal Tubular Transport of *p*-Aminohippurate by the Anterior Pituitary, 399
FARRELL, GORDON L., ROBERT C. BANKS AND SIMON KOLETSKY. The Effect of Corticosteroid Injection of Aldosterone Secretion, 104
FELDMAN, JOSEPH D. Effect of Estrus and Estrogen on Thyroid Uptake of I^{131} in Rats, 327
FLEMING, WARREN R., see MYERS, ROSE MARIE, 674
FLORES, VIOLETA, see INGLE, DWIGHT J., 388
FRAZER, M., see FARAH, A., 399
FREINKEL, NORBERT AND SIDNEY H. INGBAR. The Metabolism of I^{131} by Surviving Slices of Rat Mammary Tissue, 51
FREINKEL, NORBERT, see INGBAR, SIDNEY H., 95
FRIEDEN, EDWARD H., see NOALL, MATTHEW W., 659
FRITZ, IRVING. The Effects of the Adrenal Cortex on the Release of N^{15} from Tissue Proteins of Rats Previously Fed N^{15}-Glycine, 484
FRITZ, IRVING. The Preferential Catabolism of Recently Synthesized Protein During Starvation, 493

GILBERT, CHRISTINE AND JOSEPH GILLMAN. The Role of the Adrenal and Thyroid in Promoting Bleeding in

Hypophysectomized Baboons (Papio ursinus), 753

GILLMAN, JOSEPH, see GILBERT, CHRISTINE, 753

GLICK, DAVID AND HELEN R. STRECKLEIN. Studies in Histochemistry: Effects of Stress Conditions, ACTH, Cortisone, Desoxycorticosterone and Hypophysectomy on the Quantitative Histological Distribution of Phenolsulfatase and Acetylcholinesterase in the Rat Adrenal, 573

GLICK, DAVID, see SWIGART, RICHARD H., 582

GOLDMAN, MELVIN L., ETHEL RONZONI AND HENRY A. SCHROEDER. The Response of the Adrenal Cortex of the Rat to Dietary Salt Restriction and Replacement, 57

GOOD, THOMAS A., ROBERT S. ELY, LAWRENCE R. HEISELT, ALAN K. DONE AND VINCENT C. KELLEY. Studies of 17-Hydroxycorticosteroids: Adrenalectomy and Hypophysectomy in Guinea Pigs and their Effects on Plasma 17-OHCS Concentrations, 651

GORDON, ALBERT S., see MEGEL, HERBERT, 352

GORDON, EDWIN E. AND CLAUDE A. VILLEE. An *In Vitro* Assay for Estradiol-17β and Estrone, 150

GOULD, THELMA CLARK, see GUNN, SAMUEL A., 443

GRAFF, MORRIS M., see TULLNER, WILLIAM W., 802

GREENSPAN, FRANCIS S., JOSEPH P. KRISS, LINCOLN E. MOSES AND WILLIAM LEW. An Improved Bioassay Method for Thyrotropic Hormone using Thyroid Uptake of Radiophosphorus, 767

GREER, MONTE A. AND HOWARD L. ERWIN. Evidence of Separate Hypothalamic Centers Controlling Corticotropin and Thyrotropin Secretion by the Pituitary, 665

GREGORY, RAYMOND, L., GLENN A. DRAGER, SHIH YUAN TSAI AND LAWRENCE G. MAY. The Effect of Diabetes of Pancreactectomy on the Pituitary Cytology of Dogs, 79

GRIESER, KENNETH CHARLES AND LUDVIG G. BROWMAN. Total Gonadotrophic Potency of Mule Deer Pituitaries, 206

GUNN, SAMUEL A. AND THELMA CLARK GOULD. The Relative Importance of Androgen and Estrogen in the Selective Uptake of Zn^{65} by the Dorsolateral Prostate of the Rat, 443

HAAG, VIRGINIA, see KOCHAKIAN, CHARLES D., 315

HAGOPIAN, M., G. PINCUS, J. CARLO AND E. B. ROMANOFF. Isolation of an Unknown Substance and 6-Ketoprogesterone from Perfusates of Human Placentae, 387

HALMI, N. S., see SPIRTOS, B. N., 193

HALMI, N. S., R. G. STUELKE AND M. D. SCHNELL. Radioiodide in the Thyroid and in Other Organs of Rats Treated with Large Doses of Perchlorate, 634

HAMILTON, LYLE H., AND JULIUS LOWENTHAL. Effect of Heparin Pretreatment on Stress-Induced Leukocyte Changes in the Rat, 546

HAZELWOOD, ROBERT L., LESLIE L. BENNETT AND MARJORIE M. NELSON. Effect of Pantothenic Acid Deficiency on Insulin Sensitivity and Response to ACTH of Intact and Diabetic Rats, 427

HEAGY, F. C., see NICHOLS, DORIS, 587

HEISELT, LAWRENCE R., see GOOD, THOMAS A., 651

HELMREICH, MARY L., see SAMUELS, LEO T., 435

HERTIG, ARTHUR T., see BENIRSCHKE KURT, 598

HERTZ, ROY, see TULLNER, WILLIAM W., 282

HERTZ, ROY, see TULLNER, WILLIAM W., 802

HISAW, FREDERICK L., see BEVER, ARLEY T., 512

HOLMES, LEILA B., see SCHWARTZ, THEODORE B., 453

HOUSE, E. L., see NACE, P. F., 305

HUME, DAVID M., see NELSON, DON H., 309

IGLESIAS, R., see MARDONES, ELVIRA, 212

INGBAR, SIDNEY H., see FREINKEL, NORBERT, 51

INGBAR, SIDNEY H. AND NORBERT FRIENKEL. Concentration Gradients for Radioiodide in Unblocked Thyroid Glands of Rats: Effect of Perchlorate, 95

INGLE, DWIGHT J., GLORIA TORRALBA AND VIOLETA FLORES. Time-Response Effect of Insulin upon the Level of Plasma Amino Acids in the Functionally Eviscerated Rat, 388

JONES, JULIA C., see PORTER, JOHN C., 62

KEATING, RAYMOND F., JR., see MOLNAR, GEORGE D., 381
KEATING, F. RAYMOND, JR., see MOLNAR GEORGE D., 501
KELLEY, VINCENT C., see GOOD, THOMAS A., 651
KENYON, ALLAN T. Overture to 1956—Presidential Address, 284
KIRKWOOD, S., See SERIF, G. S., 23
KOCHAKIAN, CHARLES D., CAROL TILLOTSON AND GERALD L. ENDAHL. Castration and the Growth of Muscles in the Rat, 226
KOCHAKIAN, CHARLES D. AND W. W. EVANS. The Iodine Metabolism of the Thyroid of Androgen-Treated Rats, 279
KOCHAKIAN, CHARLES D., CAROL TILLOTSON, JANE AUSTIN, EDITH DOUGHERTY, VIRGINIA HAAG AND ROBERT COALSON. The Effect of Castration on the Weight and Composition of the Mucles of the Guinea Pig, 315
KODA, F., see FARAH, A., 399
KOLETSKY, SIMON, see FARRELL, GORDON L., 104
KORITZ, SEYMOUR B. AND RALPH I. DORFMAN. Effect of Hormones on the *In Vitro* Incorporation of Glycine-1-C^{14} into Reticulocyte Proteins, 748
KRISS, JOSEPH P., see GREENSFAN, FRANCIS S., 767

LATTANZI, W. E., see WINTERNITZ, W. W., 232
LAWRENCE, NANCY, see BAKKE, JOHN L., 531
LEVER, J. D. Cytological Studies on the Hypophysectomized Rat Adrenal Cortex: The Alterations of Its Fine Structure Following ACTH Administration and on Lowering the Na/K Ratio, 163
LEVEY, HAROLD A., ELIZABETH CHEEVER AND SYDNEY ROBERTS. Bioassay of Thyrotropin, 420
LEVITZ, MORTIMER, GEORGE P. CONDON AND JOSEPH DANCIS. The Interconversion of Estrone and Estradiol in the Perfused Human Placenta, 376
LEW, WILLIAM, see GREENSPAN, FRANCIS S., 767
LIPSCHUTZ, A., see MARDONES, ELVIRA, 212
LLAURADO, J. G. Comparison of a Radioactive Isotope Technique with Techniques Using Flame Photometry in the Bioassay of Mineralocorticoids, 390
LOEW, E. R., see DEUTSCH, S., 33
LOWENTHAL, JULIUS, see HAMILTON, LYLE H., 546
LUND, GEORGE H., see LYSTER, STANLEY C., 781
LYSTER, STANLEY C., GEORGE H. LUND AND ROBERT O. STAFFORD. Androgenic and Myotrophic Properties of Orally Administered 9-Fluoro-11-Oxy-Methyltestosterones, 781

MARDONES, ELVIRA, R. IGLESIAS AND A. LIPSCHUTZ. The Antiluteinizing Potency of Five Derivatives of Progesterone, 212
MAY, LAWRENCE G., see GREGORY, RAYMOND, L., 79
MAYER, JEAN, see SHULL, KENNETH H., 1
MAYER, JEAN, see SHULL, KENNETH H., 220
MEGEL, HERBERT AND ALBERT S. GORDON. Endocrine Influences upon Esterase Activity of Rat Bone Marrow, 352
MELTON, CARLTON E., JR. Electrical Activity in the Uterus of the Rat, 139
MEYER, ROLAND K., see BAUM, GENEVA J., 338
MEYER, ROLAND K., see CLIFTON, KELLY H., 681
MEYER, ROLAND K. AND KELLY H. CLIFTON. Effect of Diethylstilbestrol-Induced Tumorigenesis on the Secretory Activity of the Rat Anterior Pituitary Gland, 686
MIDDLESWORTH, L. VAN. Effect of Casein on Iodide Metabolism, 109
MILLER, MALCOLM R. AND DORIS H. WURSTER. Studies on the Blood Glucose and Pancreatic Islets of Lizards, 114
MILLER, RICHARD A. AND ALICE W. DOCKRELL. Evidence that the Glomerular Zone is Stimulated by Epinephrine, Causing an Eosinopinia in Hypophysectomized Rats, 550
MOE, JANET, G., see CHERNICK, SIDNEY S., 280
MOLNAR, GEORGE D., A. ALBERT, F. RAYMOND KEATING, JR. AND ALAN L. ORVIS. Iodine Metabolism in Surviving Human Canine Thyroid Slices, 381

MOLNAR, GEORGE D., F. RAYMOND KEATING, JR., ALAN L. ORVIS AND A. ALBERT. Iodine Metabolism in Surviving Thyroid Slices Maintained in a Medium of Constant Composition, 501

MOSES, LINCOLN E., see GREENSPAN, FRANCIS S., 767

MYERS, ROSE MARIE, WARREN R. FLEMING AND BRADLEY T. SCHEER. Pituitary-Adrenal Control of Sodium Flux Across Frog Skin, 674

NACE, P. F., E. L. HOUSE AND J. P. TASSONI. Alloxan Diabetes in the Hamster: Dosage and Blood Sugar Curves, 305

NALBANDOV, V., see SHIRLEY, H. V., JR., 477

NALBANDOV, A. V., see SHIRLEY, H. V., 694

NELSON, DON H., RICHARD H. EGDAHL AND DAVID M. HUME. Corticosteroid Secretion in the Adrenal Vein of the Non-Stressed Dog Exposed to Cold, 309

NELSON, MARJORIE M., see HAZELWOOD, ROBERT L., 427

NEUMANN, E. D., see BEYLER, A. L., 471

NICHOLS, DORIS, F. C. HEAGY AND R. J. ROSSITER. Phosphorus Metabolism of the Adrenal Gland: Effect of Cold Stress on the Labeling of Phospholipid and RNA Nucleotides with Radioactive Inorganic Phosphate, 587

NOALL, MATTHEW W. AND EDWARD H. FRIEDEN. Variations in Sensitivity of Ovariectomized Guinea Pigs to Relaxin, 659

Obituary of Carl R. Moore, 529

ORVIS, ALAN L., see MOLNAR, GEORGE D., 381

ORVIS, ALAN L., see MOLNAR, GEORGE D., 501

PAULY, JOHN E., see ELIAS, HANS, 714

PINCUS, G., see HAGOPIAN, M., 387

PORTER, JOHN C. AND JULIA C. JONES. Effect of Plasma from Hypophyseal-portal Vessel Blood on Adrenal Ascorbic Acid, 62

PORTER, JOHN C. AND RUMSFELD, H. W., JR. Effect of Lyophilized Plasma and Plasma Fractions from Hypophyseal-Portal Vessel Blood on Adrenal Ascorbic Acid, 359

POSTEL, SHOLEM. Zone Electrophoresis in the Preparation of Serum and Pituitary Extracts for Bioassay of Thyroid Stimulating Hormone, 557

POTTER, G. D., see TAUROG, ALVIN, G., 132

ROBERTS, SYDNEY, see LEVEY, HAROLD A., 420

ROBERTSON, MARY C., see SCHWARTZ, THEODORE B., 453

ROMANOFF, E. B., see HAGOPIAN, M., 387

RONZONI, ETHEL, see GOLDMAN, MELVIN, L., 57

ROSEMBERG, EUGENIA, see ROSENFELD, GEORGE, 255

ROSEMBERG, E., see BLOCH, E., 626

ROSEMBERG, EUGENIA, GEORGE ROSENFELD, FRANK UNGAR AND RALPH I. DORFMAN. Conversion of Steroids to Aldosterone-like Material, 708

ROSENFELD, GEORGE, EUGENIA ROSEMBERG, FRANK UNGAR AND RALPH I. DORFMAN. Regulation of the Secretion of Aldosterone-Like Material, 255

ROSENFELD, GEORGE, see ROSEMBERG, EUGENIA, 708

ROSSITER, R. J., see NICHOLS, DORIS F., 587

RUGH, ROBERTS AND HELEN CLUGSTON. Effect of Potassium Thiocyanate on Fetal Retention of Radioiodine, 8

RUMSFELD, H. W., JR., see PORTER, JOHN C., 359

RUSSELL, JANE A. AND WALTER BLOOM. Hormonal Control of Glycogen in the Heart and Other Tissues in Rats, 83

SAMUELS, LEO T. AND MARY L. HELMREICH. The Influence of Chorionic Gonadotropin on the 3β-OL Dehydrogenase Activity of Testes and Adrenals, 435

SAUNDERS, FRANCIS J. Survival of Adrenalectomized Mice After Several Types of Stress and the Effects of Various Steroids, 412

SAUNDERS, FRANCIS J. AND VICTOR A. DRILL. The Myotrophic and Androgenic Effects of 17-Ethyl-19-Nortestosterone and Related Compounds, 567

SCHEER, BRADLEY T., see MYERS, ROSE MARIE, 674

SCHMIDT-NIELSEN, BODIL, see ADAMSONS, KARLIS, JR., 272

SCHMIDT-NIELSEN, KNUT, see ADAMSONS, KARLIS, JR., 272
SCHNELL, M. D., see HALMI, N. S., 634
SCHROEDER, HENRY A., see GOLDMAN, MELVIN L., 57
SCHWARTZ, THEODORE B., MARY C. ROBERTSON AND LEILA B. HOLMES. Adrenocortical Influences on Dipeptidase Activity of Surviving Rat Diaphragm, 453
SELYE, HANS AND PIERRE BOIS. Indispensability of the Adrenals for the Nephrosclerotic Action of Hypophyseal Preparations, 739
SERIF, G. S. AND S. KIRKWOOD. The Mechanism of the Antithyroid Action of Iodide Ion, 23
SHELLABARGER, C. J. Studies on the Thyroidal Accumulation of Rhenium in the Rat, 13
SHELLABARGER, C. J. The Effect of n-Methyl-Phenyl-Ethyl Barbituric Acid on Thyroid Function in the Rat, 347
SHIRLEY, H. V., JR. AND A. V. NALBANDOV. Effects of Neurohypophysectomy in Domestic Chickens, 477
SHIRLEY, H. V. JR. AND A. V. NALBANDOV. Effects of Transecting Hypophyseal Stalks in Laying Hens, 694
SHULL, KENNETH H. AND JEAN MAYER. Analysis of the Blood Sugar Response of Obese-Hyperglycemic Mice and Normal Mice to Hormones: Growth Hormone, Cortisone, and Corticotropin, 1
SHULL, KENNETH H. AND JEAN MAYER. Analysis of the Blood Sugar Response of Obese-Hyperglycemic Mice and Normal Mice to Hormones: Insulin, Glucagon, and Epinephrine, 220
SPIRTOS, B. N. AND N. S. HALMI. Influence of Somatotrophin and Cortisone on the Insulin Sensitivity of Rats, 193
STAFFORD, ROBERT O., see LYSTER, STANLEY C., 781
STECKLEIN, HELEN R., see GLICK, DAVID, 573
STECKLEIN, HELEN R., see SWIGART, RICHARD H., 582
STUELKE, R. G., see HALMI, N. S., 634
SWIGART, RICHARD H., HELEN R. STECKLEIN AND DAVID GLICK. Studies in Histochemistry: Effects of ACTH, Cortisone, and Desoxycorticosterone on the Quantitative Histological Distribution of Potassium in the Monkey and Guinea Pig Adrenal, 582
TASSONI, J. P., see NACE, P. F., 305
TAUROG, ALVIN, JOHN D. WHEAT AND I. L. CHAIKOFF. Nature of the I^{131} Compounds Appearing in the Thyroid Vein After Injection of Iodide-I^{131}, 121
TAUROG, ALVIN, G. D. POTTER, WINTON TONG, I. L. CHAIKOFF. The Formation of I^{131}-Monoidotyrosine from I^{131}-Iodide by Isolated Particulate Fractions of Non-Thyroid Tissues, 132
TILLOTSON, CAROL, see KOCHAKIAN, CHARLES D., 226
TILLOTSON, CAROL, see KOCHAKIAN, CHARLES D., 315
TIMIRAS, PAOLA S. AND DIXON M. WOODBURY. Effect of Thyroid Activity on Brain Function and Brain Electrolyte Distribution in Rats, 181
TONG, WINTON, see TAUROG, ALVIN, G., 132
TORRALBA, GLORIA, see INGLE, DWIGHT J., 388
TSAI, SHUH YUAN, see GREGORY, RAYMOND L., 79
TULLNER, WILLIAM W. AND ROY HERTZ. The Effect of 17 Alpha-Hydroxy-11-Desoxycorticosterone on Estrogen-Stimulated Chick Oviduct Growth, 282
TULLNER, WILLIAM W., MORRIS M. GRAFF AND ROY HERTZ. Amphenone Inhibition of Adrenal Corticosteroid Output in the Hypophysectomized Dog, 802

UNGAR, FRANK, see ROSENFELD, GEORGE, 255
UNGAR, FRANK, see ROSEMBERG, EUGENIA, 708

VAN DYKE, H. B., see ADAMSONS, KARLIS, JR., 272
VAN MIDDLESWORTH, L. Effect of Casein on Iodide Metabolism, 109
VAN MIDDLESWORTH L. A Method for Iodide Balance Studies in Animals on Low Iodide Diets, 235
VELARDO, JOSEPH T., see BEVER, ARLEY T., 512
VILLEE, CLAUDE A., see GORDON, EDWIN E., 150

WARREN, ROSLYN PAUKER AND LESTER R. ARONSON. Sexual Behavior in Castrated-Adrenalectomized Hamsters Maintained on DCA, 293

WEBER, GEORGE, CLAUDE ALLARD, GASTON DE LAMIRANDE AND ANTONIO CANTERO. Liver Glucose-6-Phosphatase Activity and Intracellular Distribution after Cortisone Administration, 40

WHEAT, JOHN D., see TAUROG, ALVIN, 121

WINTERNITZ, W. W. AND W. E. LATTANZI. Liver Glycogen Reserves in Experimental Diabetic Ketosis, 232

WOODBURY, DIXON, M., see TIMIRAS, PAOLA S., 181

WURSTER, DORIS H., see MILLER, MALCOLM R., 114

SUBJECT INDEX, VOLUME 58

Adrenal cortex
response to dietary salt restriction and replacement, 57
effect of hypophyseal portal vessel blood plasma and plasma fractions on adrenal ascorbic acid, 62, 359
cytological changes induced by ACTH and altered Na/K ratio in hypophysectomized rats, 163
corticosteroid levels in adrenal vein of non-stressed dog exposed to cold, 309
action on bone marrow esterase, 352
diurnal variation in blood leucocytes in normal and adrenalectomized mice, 365
comparison of a radioactive isotope technique with others in bioassay of mineralocorticoids, 390
survival of adrenalectomized mice after stress and administration of steroids, 412
influence on dipeptidase activity of surviving rat diaphragm, 453
action on release of N^{15} from tissue proteins of rats previously fed N^{15} glycine, 484
preferential catabolism of recently synthesized protein during starvation, 493
evidence that epinephrine stimulates glomerular zone causing eosinopenia in hypophysectomized rats, 550
hormones on histological distribution of potassium in monkey and guinea pig adrenal, 582
hormones on histochemical distribution of sulfatase and acetylcholinesterase in rat adrenal, 573
phosphorus metabolism of cortex during cold stress, 587
concerning function of fetal zone in human, 598
steroids in human fetal adrenal glands, 626
plasma 17-hydroxycorticosteroids in adrenalectomized and hypophysectomized guinea pigs, 651
non-effect of ascorbic acid pretreatment on response of, 671
control of sodium flux across frog skin, 674
structure of human adrenal cortex, 714
importance of nephrosclerotic action of hypophyseal preparations, 739
influence of steroids on reticulocyte protein synthesis, 748
role in promoting bleeding in hypophysectomized baboons, 753
Adrenal medulla
effect on muscle glycogen in normal and hypophysectomized rats, 158
Aldosterone
effect of corticosteroids on secretion of, 104
regulation of the secretion of aldosterone-like material, 255
conversion of steroids to aldosterone-like material, 708
Amphenone
inhibition of adrenal corticosteroid output in hypophysectomized dog, 802
Androgens
castration and growth of muscles in rats, 226
effect on iodine metabolism, 279
effect of castration on weight and composition of the muscles of the guinea pig, 315
action on selective uptake of Zn^{65} by dorsal prostate in rat, 443
myotrophic and androgenic effects of 17-ethyl-19-nortestosterone and related compounds, 567
influence on *in vitro* incorporation of glycine-1-C^{14} into reticulocyte protein, 748
androgenic and myotrophic properties of 9-fluoro-11-oxymethyltestosterones, 781

Chorionic gonadotropin
effect on the 3β-OL dehydrogenase activity of testes and adrenals, 435
Corticotropin
effect on blood sugar in obese hyperglycemic mice, 1
effect of pantothenic acid deficiency on

insulin sensitivity and response to ACTH of intact and diabetic rats, 427
evidence for separate hypothalamic center controlling pituitary secretion of, 665•
influence of oxycel corticotropin on fatty acid and glucose metabolism in rats, 808
Cortisone
effect on blood sugar in obese mice, 1
effect on liver glucose-6-phosphatase, 40
action on insulin sensitivity in rats, 193

Epinephrine
effect on blood sugar in normal and obese mice, 220
action on glomerular zone of hypophysectomized rat adrenal in relation to induced eosinopenia, 550
Estrogens
an *in vitro* assay of estradiol-17β and estrone, 150
effect on thyroid uptake of I^{131} in rats, 327
effect of diethylstilbestrol on lipids in cockerels, 338
interconversion of estrone and estradiol in the perfused human placenta, 376
action on lactic dehydrogenase-DPNH Oxidase System of Rat Uterus, 512
effect of diethylstilbestrol-induced tumorigenesis on the secretory activity of the rat anterior pituitary gland, 686
glycine-1-C^{14} into reticulocyte protein, 748
Estrus
effect on thyroid uptake of I^{131} in rats, 327

Glucagon
effect on blood sugar in normal and obese mice, 220
Gonadotrophins
potency of mule deer pituitaries, 206
antilutenizing potency of derivatives of progesterone, 212
Growth hormone
effect on blood sugar in obese-hyperglycemic mice, 1
control of glycogen in heart and other tissues of rat, 83
influence on insulin sensitivity in rats, 193
action on bone marrow esterase, 352
selective effects of growth hormone on rats fed a diet low in tryptophan, 701
a comparison with insulin administration, 797

Hypophyses
effect of pancreatectomy in dogs on cytology of, 79
effect of removal on coenzyme content of rat liver, 280
control of renal tubular transport of p-aminohippurate, 399
effect of transecting stalk in laying hens, 694
steroid induced adrenal-pituitary hypofunction, 777
functional corpora lutea maintained by autografts of rat hypophysis, 786
Hypothalamus
endocrine influence on antidiuretic activity of rat hypothalamic-neurohypophyseal system, 461
evidence for separate centers for control of pituitary secretion of TSH and ACTH, 665

Insulin
effect on blood sugar in normal and obese mice, 220
time-response effect of insulin upon the level of plasma amino acids in the eviscerated rat, 388
Iodine
metabolism by mammary tissue *in vitro*, 51

Mammary gland
metabolism of I^{131} by surviving slices of, 51
Melanophore expanding hormone
quantitative method for measuring, 33

Neurohypophysis
distribution of oxytocin and vasopressin (ADH) in neurohypophysis of camel, 277
endocrine influences on antidiuretic activity of rat hypothalamic-neurohypophyseal system, 461
ablation effect in fowl, 477
effect of transecting stalk in laying hens, 694

Pancreas
studies on blood glucose and islet tissue of lizards, 114
liver glycogen reserves in experimental diabetic ketosis, 232
alloxan diabetes in the hamster, 305

Placenta
interconversion of estrone and estradiol in the perfused human placenta, 376
isolation of an unknown substance and 6-ketoprogesterone from perfusates of human placenta, 387

Relaxin
variation in sensitivity of ovariectomized guinea pigs to, 659

Reproductive system
effect of steroid hormones in pullets on development of, 262
effect of 17 alpha-hydroxy-11-desoxycorticosterone on estrogen stimulated chick oviduct growth, 282

Sexual behavior
in castrated-adrenalectomized hamsters maintained on DCA, 293

Testes
castration and growth of muscles in rats, 226
effect of castration on weight and composition of the muscles of the guinea pig, 315
effect on the 3β-OL dehydrogenase activity, as influenced by chorionic gonadotropin, 435

Thyroid gland
effect of potassium thiocyanate on fetal retention of radioiodine, 8
accumulation of rhenium, 13
mechanism of antithyroid action of iodide ion, 23
extra-thyroidal iodide metabolism in rat, 68
action of perchlorate on concentration gradients for radioiodide in unblocked thyroid glands of rats, 95
effect of casein on iodide metabolism, 109
nature of I^{131} compounds in thyroid vein after injection of iodide I^{131}, 121
formation of I^{131} monoiodotyrosine from I^{131} iodide by isolated particulate fractions of non-thyroidal tissues, 132
action on brain function and brain electrolyte distribution in rats, 181
a method for iodide balance studies in animals on low iodide diets, 235
iodine metabolism of the thyroid of androgen-treated rats, 279
effect in rats of n-methyl-phenyl-ethyl barbituric acid on function of, 347
iodine metabolism in surviving human and canine thyroid slices, 381, 501
radioiodide in thyroid and other organs of rats treated with perchlorate, 634
role in promoting bleeding in hypophysectomized baboons, 753

Thyrotropin
Bioassay of, 420
effect on iodide collecting mechanism of thyroid tissue slices, 531
zone electrophoresis in preparation of serum and pituitary extracts for bioassay of, 557
evidence for a separate hypothalamic center controlling pituitary secretion of, 665
secretion as altered by food intake during treatment with diethylstilbestrol, 681
thyroid uptake of radiophosphorus as an improved bioassay method for, 767

Uterus
electrical activity in rat, 139

Water metabolism
endocrine factors in water metabolism of the desert mammal *G. gerbillus*, 243

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(Continued on page iv)

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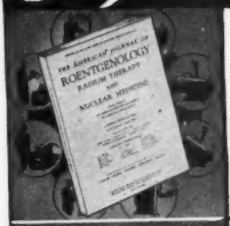

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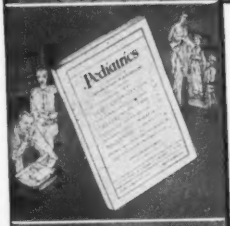

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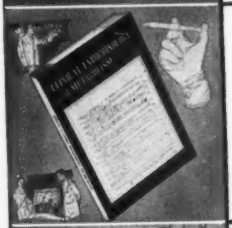

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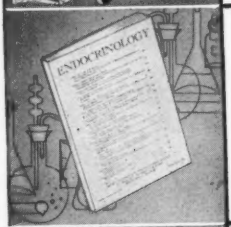

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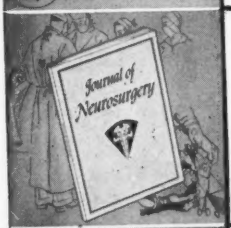

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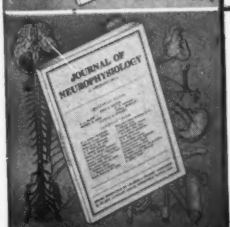

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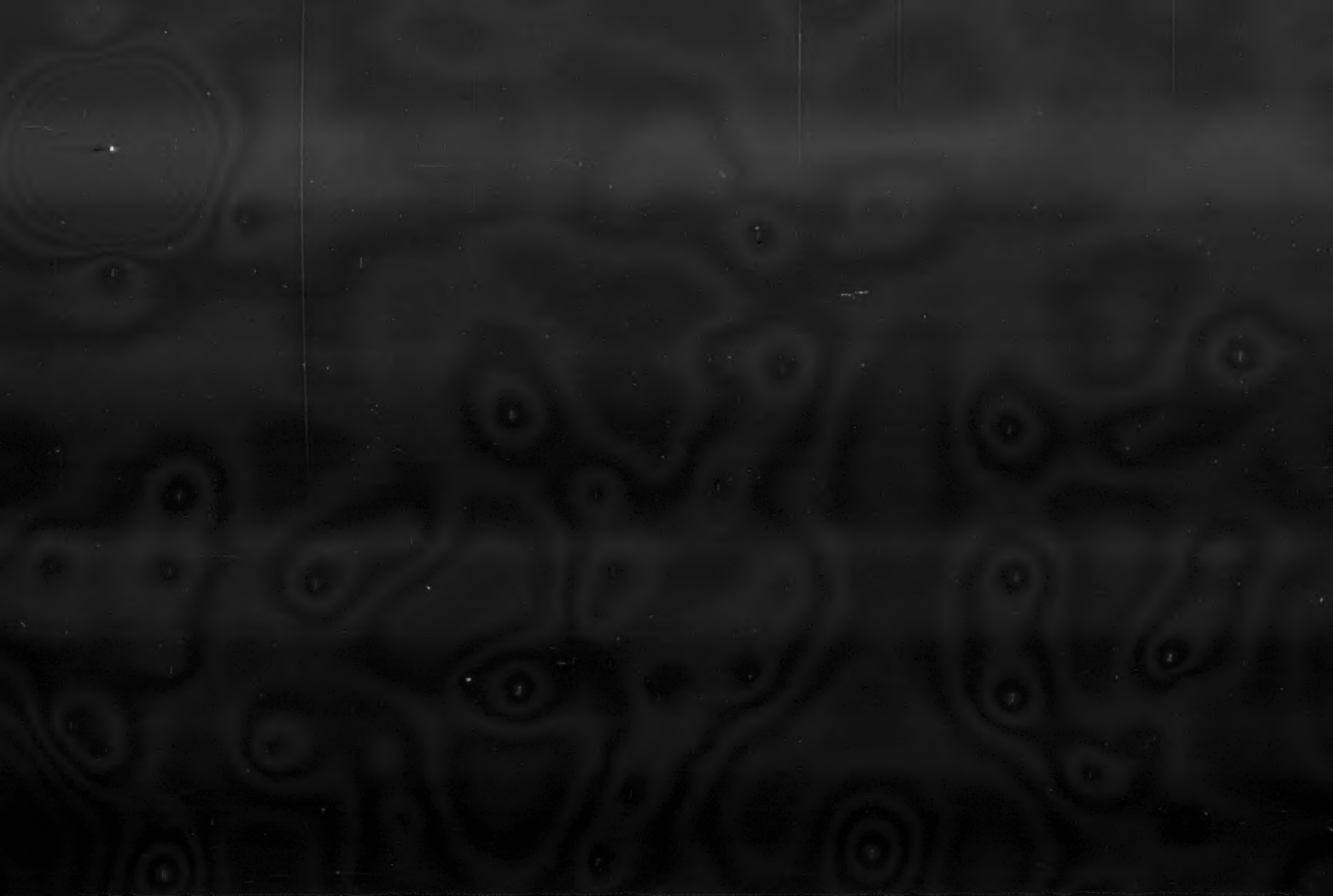

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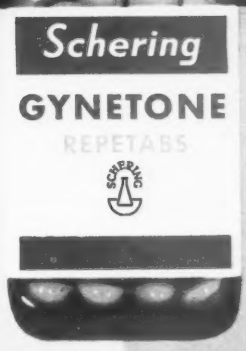

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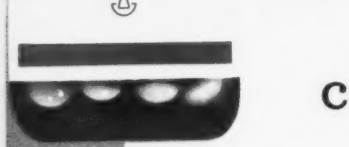

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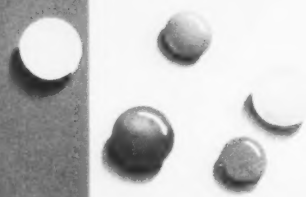

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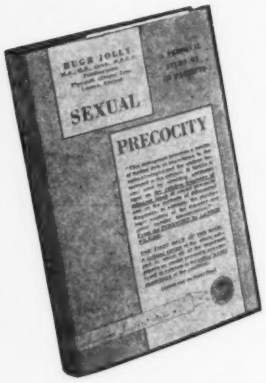

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